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EDITOR-IN-CHIEF
**Oscar Kuang-Sheng Lee, MD, PhD, Professor,** Medical Research and Education of Veterans General Hospital-Taipei, No. 322, Sec. 2, Shih-pai Road, Shih-pai, Taipei 11217, Taiwan

EDITORIAL OFFICE
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*World Journal of Stem Cells*
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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PUBLISHER
Baishideng Publishing Group Inc
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EDITORIAL

# Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine

Pedro Manuel Aponte

Pedro Manuel Aponte, AGROCALIDAD/Project Prometeo - SENESCYT, Quito 170184, Ecuador
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Correspondence to: Pedro Manuel Aponte, DVM, PhD, Scientific Adviser at AGROCALIDAD/Project Prometeo - SENESCYT, Av. 9 de Octubre N22-48 y Jerónimo Carrión, Casa Patrimonial, Quito 170184, Ecuador. apontep@gmail.com
Telephone: +593-2-2372844
Fax: +593-2-2372844
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## Abstract

Spermatogonial stem cells (SSCs) are the germ stem cells of the seminiferous epithelium in the testis. Through the process of spermatogenesis, they produce sperm while concomitantly keeping their cellular pool constant through self-renewal. SSC biology offers important applications for animal reproduction and overcoming human disease through regenerative therapies. To this end, several techniques involving SSCs have been developed and will be covered in this article. SSCs convey genetic information to the next generation, a property that can be exploited for gene targeting. Additionally, SSCs can be induced to become embryonic stem cell-like pluripotent cells *in vitro*. Updates on SSC transplantation techniques with related applications, such as fertility restoration and preservation of endangered species, are also covered on this article. SSC suspensions can be transplanted to the testis of an animal and this has given the basis for SSC functional assays. This procedure has proven technically demanding in large animals and men. In parallel, testis tissue xenografting, another transplantation technique, was developed and resulted in sperm production in testis explants grafted into ectopical locations in foreign species. Since SSC culture holds a pivotal role in SSC biotechnologies, current advances are overviewed. Finally, spermatogenesis *in vitro*, already demonstrated in mice, offers great promises to cope with reproductive issues in the farm animal industry and human clinical applications.

**Key words:** Cell culture; Spermatogonial stem cells; Spermatogenesis; Pluripotency; Transplantation; Fertility

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**Core tip:** This article reviews the current body of knowledge on biotechnological applications of spermatogonial stem cells (SSCs). SSCs are the founding adult germ stem cells of the sperm producing process spermatogenesis. SSCs belong to the male germline and can be expanded *in vitro* in several species. Through mechanisms not fully understood they can derive pluripotent stem cells *in vitro*. Thus, they can be genetically modified with some advantages over embryonic stem cell-based technologies. SSCs can be transplanted to homotopical or ectopical locations, offering great potentials in fertility related issues and regenerative clinical applications in domestic or wild animals and men.

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## INTRODUCTION

Spermatogonial stem cells (SSCs), the germ stem cells of the seminiferous epithelium in the testis are the founder cells of a sperm producing stem cell system called spermatogenesis. SSCs were initially regarded as unipotential cells. Nowadays, however, current knowledge about SSCs has expanded to new limits and the general concept of SSCs as cells just supporting the production of the terminally differentiated sperm has rather radically changed. As a result of continuous and elaborate SSC biology research, the manipulation of this special cell type offers novel biotechnological solutions to contemporary human problems. SSCs are the only adult stem cells capable of transmitting the genome of a given species from one generation to the next, while at the same time having the capacity to convert into pluripotent stem cells[1-3]. They can also give rise to cells from the basic three embryonic layers *in vitro*, which opened new opportunities for regenerative medicine[1]. Since SSCs are adult cells, they do not bring about ethical concerns as occurs with the use of embryonic stem (ES) cells. However, the use of SSCs is not without risk, since they show some issues regarding epigenetic inheritance, as such a topic will be covered later on this article.

The faculty of SSCs to deliver genes over generations makes them a valuable vehicle for transgenesis techniques. This is particularly important for gene targeting in domestic animals, in which, contrary to laboratory rodent species, the techniques involving SSCs are still not feasible. In this process, SSC culture plays a central role in the exploitation of the unique properties of SSCs for biotechnological applications. Historically, the characterization of the SSC niche *in vivo* gave the basis for SSC culture conditions, especially SSC population expansion *via* self-renewal in several species, included men. A stem cell niche consists of a microenvironment with supporting cells and a corresponding signal exchange that regulates self-renewal and differentiation of the stem cells. Accordingly, SSCs are located in the seminiferous epithelium, near the basement membrane and the niche environment normally contains factors produced by neighboring somatic cells (sertoli cells, leydig cells, and peritubular myoid cells), the basement membrane and in particular the vascular network, near where SSCs are preferentially located[4]. This microenvironment in the testis provides signals to control stem cell self-renewal, differentiation, or survival, guaranteeing a normal cell kinetics in spermatogenesis[5,6].

SSC behavior *in vitro* could be further investigated thanks to the development of germ cell transplantation assays, which allow functional verification of the presence of SSCs (see the topic Germ cell transplantation). The addition of certain growth factors to the culture media, in particular glial cell line-derived neurotrophic factor (GDNF), and the availability of a functional SSC assay, allows SSC populations to be expanded during long term culture, an advance first made in rodent models.

SSC biotechnologies are being aimed to solve fertility related issues (*i.e.*, fertility restoration in oncological patients, infertility treatments and reproduction of endangered species). They also provide important developments in germplasm preservation and the emerging technology of spermatogenesis *in vitro*. Finally, several of these applications require species-specific knowledge of SSC culture conditions as well as transplantation techniques, two aspects that will be covered in this article (for a summary of the biotechnological approaches that involve the use of SSCs, see Figure 1).

## SSC ISOLATION AND CULTURE

SSC isolation procedures have been described for several domestic species and humans[7-14]. SSCs are isolated by enzymatic digestion, usually involving two steps of various combinations of proteolytic enzymes (commonly, collagenases Ⅰ or Ⅳ, trypsin, hyaluronidase and DNAse Ⅰ). From this enzymatic digestion, a germ cell suspension is obtained that now has to go through at least one type of purification methods, but more often a combination of them (Zheng *et al*[15], 2014 summarize the current enriching methods for SSC isolation). There are two groups of purification methods, one group based on the SSC phenotype, like fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS), and the other utilizes physical methods, like differential plating, extracellular matrix selection and density gradient sedimentation[15]. Simple models for SSC isolation include the use of young animals[16], cryptorchid animals[17], vitamin A deficient animals[18], but SSC isolation becomes more complex when their source is the adult testis with full spermatogenesis. In this scenario, all differentiating germ cell types are present and SSCs represent a smaller proportion (see the topic SSC markers). Most recent articles in which SSC isolation is carried on, include one phenotype-based method and one or more physical methods. Most researchers do not use a functional assay (germ cell transplantation) to unambiguously identify the isolated germ cells as SSCs. They instead characterize the germ cell colonies arising during culture by means of immunohistochemistry or RT-PCR, aiming to identify gene products or RNA expression respectively (see the topic SSC markers). MACS is the most widely used technique nowadays. It is simple and inexpensive, but relies on specific SSC markers usually present in subsets of the SSCs, therefore sub-sampling the

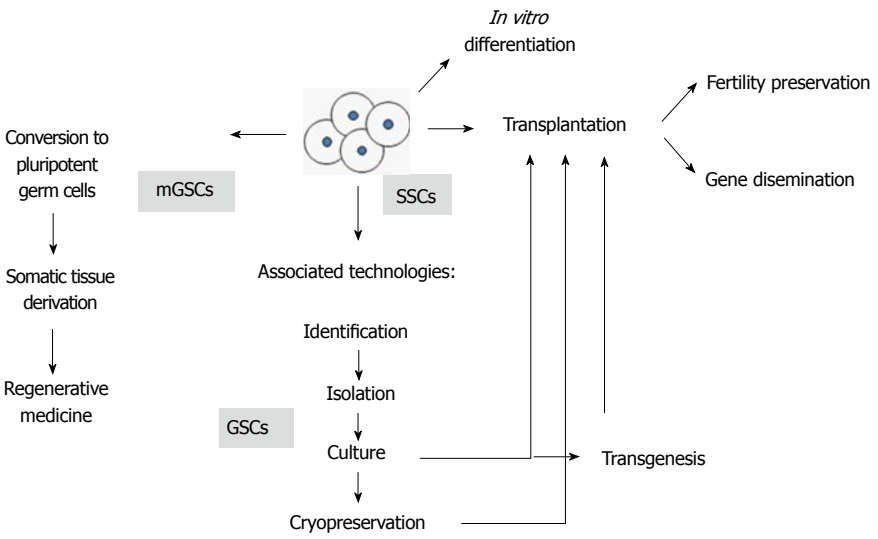


**Figure 1 Overview of biotechnologies involving spermatogonial stem cells.** Fertility preservation includes fertility restoration in oncological patients after therapeutic agents are administered that produce germ cell damage, strategies for infertile male patients and reproduction of endangered species. Gene dissemination represents the possibility to use several low-genetics animals to disseminate elite genes in animal production operations. Culture involves maintenance, survival, expansion and possibly genetic manipulations of spermatogonial stem cells (SSCs). Germ stem cells (GSCs), that is, SSCs that self-renew in vitro, therefore proliferating and expanding. Multipotent germ stem cells (mGSCs), which spontaneously appear in culture from within the SSC population, showing pluripotency properties[2].

general SSC population. Interestingly, one group, in a recent work, produced an enriched human SSC suspension by using MACS and the marker ITGA6 (alpha 6 integrin) together with a proper authentication of SSCs through the germ cell transplantation assay[11]. A recent novel approach for SSC isolation involved the combination of traditional isolation methods with testicular tissue grafting (see the topic Germ cell transplantation). In this work, adult mouse testis tissue was grafted into the dorsal skin of immunodeficient mice and after 2-4 wk the researchers observed the depletion of advanced differentiating germ cells of the seminiferous epithelium. Afterwards, the tissues left only with undifferentiated type A spermatogonia (a population including the SSCs) were recovered. The SSCs were isolated from the tissues, which were not only able to proliferate *in vitro* but also to recapitulate spermatogenesis in receptor testes devoid of endogenous spermatogenesis and furthermore to produce offspring through the original donor derived sperm[19]. This method, although perhaps not as practical as MACS, results in a larger true SSC population since the whole array of markers confirm the phenotypic profiles of SSCs.

Culture conditions for SSC propagation *in vitro* were first developed in mice[16,20]. This was the base for the development of domestic animals SSC culture systems[7-10,21-25]. Most of these systems have in common the use of GDNF and serum. Common growth factors, besides GDNF, include LIF, FGF2 and EGF[26], however important responses of SSCs regarding maintenance, survival and growth *in vitro* are dependent on age, species and even the strain of the donor animals[27]. Very recently, a feeder/serum-free culture was established for mice SSCs[28] and this method was improved for maintenance of SSCs[29]. This represents a big advance because serum, when present, introduces unknown variable factors into the culture systems[27] besides promoting the growing of contaminating somatic cells[2]. Current efforts are being made to set up functional human SSC culture systems[11,30,31].

## SSC DIFFERENTIATION *IN VITRO*

Normally, SSCs self-renew during the steady state cell kinetics of spermatogenesis. They do so to maintain the undifferentiated pool while at the same time producing daughter cells that enter the differentiation path, ultimately generating sperm[32]. Thus, the production of sperm *in vitro* requires culture conditions favoring the pathways of sperm production and not self-renewal. Going through the meiosis step of germ cell differentiation *in vitro* was one of the most difficult steps for many research groups. This fact hindered the progress in this particular research area for many years. An spermatogenesis *in vitro* system going beyond that developmental stage and traversing spermiogenesis was achieved and mouse sperm could be generated when testicular tissue was set on the liquid/gas interphase[33]. This system included a very simple culture medium and testicular tissues came from young animals[34]. One of the main features of this culture system is the preservation of the cytoarchitecture of the testis, that is, maintaining the structure of SSC niches. The reconstitution of spermatogenesis *in vitro* from SSC suspensions has proven more challenging. Some groups have obtained sperm *in vitro* by using testicular cell suspensions from prepubertal animals but three dimensional support matrices are necessary[35,36]. Although these successful systems have been developed for sperm production

*in vitro* with rodent species, it is not yet foreseeable that these techniques can be translated to a clinical setting in the near future. Human SSC differentiation in culture systems is still under investigation at the present time.

The exciting possibility of producing sperm *in vitro* has large implications for farm animal industries, human fertility restoration, preservation of endangered species and many other associated technologies related to mammalian reproduction. For instance, in the cattle industry, keeping animals in large facilities would be a thing of the past when renewable SSC pools from elite bulls produce high numbers of sperm in Petri dishes at small biotechnological facilities. In many cases of human infertility it would just be enough to have one healthy SSC derived (and possibly engineered) sperm to produce offspring through assisted reproduction technologies (ARTs). Autologous SSC culture systems could be set for individuals before oncological treatments and sperm generated *in vitro* could be cryopreserved for later use to fertilize an egg and produce offspring from these patients. This way, the danger of reintroducing malignant cells back into the patient could be avoided. SSC banking could then become possible, as another mean for germplasm preservation in many species. Moreover, the cumbersome step of transplanting genetically modified SSCs to a receptor male in order to recapitulate spermatogenesis and thus obtain transgenic sperm could then be omitted. In addition, these techniques could also provide means for preserving endangered species, for instance, animal populations with very few individuals that at the moment of dying could have their SSCs collected for sperm to be produced in the laboratory.

## SSC MARKERS

The characterization of SSCs is an important tool to study their presence on culture systems and during other techniques. SSCs, representing only the 0.03% of germ cells in the testis[37], and the more numerous type A spermatogonia committed to differentiate, are morphologically undistinguishable. Furthermore, there is no general consensus on the phenotypic profile of SSC as this may vary among different species[38] and there seems to be no unique SSC marker. In general, some phenotypic characteristics of SSCs have been advanced thanks to the molecular dissection of the SSC niche in the testis. Within the testis, Sertoli cells are the main cell type involved in the SSC niche[39]. The key factor GDNF, secreted by Sertoli cells, promotes SSC proliferation[40,41]. Any receptors expressed in SSCs that bind self-renewal promoting ligand molecules from the niche define their stem cell phenotype and constitute potential SSC markers[42]. That is the case of the widely accepted marker GFR$\alpha$1, the receptor for GDNF in SSCs[43]. In domestic animals, besides GFR$\alpha$1, another important marker for both gonocytes (perinatal germline stem cells) and SSCs is THY1[44-46]. In monkeys, SSCs express the phenotype (CD90+ DC49f$^+$ CD117$^-$), SSEA-4$^+$ PLZF$^+$[47]. Apparently, SSEA-4 seems to be conserved in humans[48]. SSCs from humans also express GPR125[49], besides GFR$\alpha$1[50]. Another human SSC marker that adds to the known panel is ITGA6[51]. Recently, a new SSC gene product has been reported: PAX7, that might represent a definite common SSC marker for several species including mice, cats, dogs, pigs, bulls, monkeys and humans[52].

## SSC PLURIPOTENCY

Pluripotent stem cells so far considered for regenerative medicine strategies include inner cell mass derived ES cells[53,54], somatic derived induced pluripotent stem cells (iPS cells)[55], fetal derived embryonic germ (EG) cells[56] and SSC derived multipotent germ stem (mGS) cells[57,58]. All of these cells form teratomas when injected to seminiferous tubules or subcutaneous tissue of immunocompromised mice and contribute to chimera formation when injected to a blastocyst. When SSCs are placed in culture they proliferate in the presence of GDNF. This growth factor is produced by Sertoli cells in the testis, stimulating SSCs to self-renew[41]. SSCs proliferating in culture are designated germ stem cells (GS) cells[16]. They are unipotent, genetically and epigenetically stable, do not produce teratomas and do not contribute to chimera[59]. Extraordinarily, while in culture, they can spontaneously derive multipotent germ stem cells (designated mGS cells) at a low frequency (1 out of 30 testes)[58]. They represent SSCs with pluripotency, sharing not only ES cell traits such as self-renewal properties, tumorogenicity and chimera production but also genomic and epigenetic abnormalities[2]. Pluripotent germ cells from the testis can circumvent some of the typical objections or technical limitations of other pluripotent cells (*i.e.*, ES cells and iPS cells) for their use in therapeutic purposes. These limitations include issues in cell proliferation, cell differentiation, genetic stability, allogenicity and ethical/religious issues (reviewed in[60]). Remarkably and unlike other pluripotent cells, the conversion of GS cells into mGS cells occurs on a dedifferentiation process not dependent on genetic manipulations, which opens important theoretical possibilities for these technologies to be translated into clinical applications in regenerative medicine. Nevertheless, despite all the promising traits of mGS cells, caution is required on the grounds of their genetic and epigenetic behavior. Although GS cells maintain a normal karyotype, genomic imprinting, and SSC activity during long-term culture[59], mGS cells are unstable and tend to accumulate genomic and epigenomic abnormalities[2,61]. Also, GS-like cells derived from mouse fetal germ cells had SSC activity but some of the produced offspring had abnormal genomic imprinting patterns, which were also transmitted to future generations[56]. SSC-derived pluripotent cells

are able to generate very diverse tissues while in cell culture[1,3]. For instance, differentiated tissues appear in culture from a specific subpopulation of mouse mGS cells that show a pluripotent cell phenotype (POU5F1$^+$) while at the same time expressing SSC proteins (c-Kit$^+$)[62].

All in all, SSCs show excellent perspectives for applications in regenerative medicine. According to the "organ niche" theory, developmental failure in organogenesis caused by defective genes, can be compensated by xenogeneic pluripotent stem cell transplantation into genetically affected blastocysts[63]. The pluripotential cells would then integrate into the embryo, resulting in chimera formation with colonization of stem cells of xenogeneic origin in the organ forming niches during development. This has been demonstrated through animal models such as mouse and pigs to successfully generate functional kidneys[64] and pancreas[63,65]. SSC-derived pluripotent stem cells, with all their inherent advantages, could be used for these purposes.

Although the current advances in the generation of testicular pluripotent cells in rodents are unquestionable, similar results in the humans have been controversial. Although one work demonstrated teratoma formation and pluripotency markers expression[66] on these cells, tumors showed a relatively small volume[67]. Several other groups have produced allegedly human mGS cells as well, but these cells, although expressing several pluripotency markers, do not produce teratomas when transplanted into the skin of immunocompromised mice[68-70]. In fact, one group recently found that human testicular embryonic-like cells (allegedly mGS pluripotent cells) do not possess a transcriptome similar to that of human ES cells but rather have the phenotype of mesenchymal stem cells (MSCs), therefore, concluding that these cells are not pluripotent and most likely not of germ cell origin but instead mesenchymal[71]. Current knowledge of human SSC culture conditions probably is insufficient and do not emulate the mouse SSC culture environment that has allowed the generation of mGS cells in this species. For further progress to be made, the exact mechanisms of SSC reprogramming need to be addressed. For instance, we know that primordial germ cells from the fetal period convert to ES-like pluripotent cells[72] *via* the AKT signaling pathway[73]. A very recent study found that spontaneous SSC reprogramming would be caused by unstable DNA methylation with involvement of the Dmrt1-Sox2 signaling pathway[74]. However, more research is required to successfully obtain human mGS cells. This will require very specific culture conditions and the molecular dissection of SSC pluripotency, all of which should continue to be investigated.

## GENE TECHNOLOGIES AND SSC

ES cell-based transgenesis technologies are well developed and routinely used in the context of mouse species. However, SSCs can be used for a more straightforward route to transgenesis. ES cells, after genetically intervened, are injected into blastocysts with the hope of successful integration and contribution to the germline, which not always can be guaranteed. By contrast, SSCs are already constituents of the germline. Some other important differences with ES cells make SSCs best suited for mutagenesis (Table 1), especially in species for which ES technologies are not feasible or physiological limitations exist like in farm animals. Additionally, unlike mice, some farm animals, primates and humans are uniparous species as a consequence of not producing large numbers of oocytes under normal physiological conditions. In addition, these species go through lengthy periods of time to attain puberty and sexual maturity. SSC genetic and epigenetic stability keep these cells committed to the germline phenotype so that they do not tend to differentiate into other lineages[59]. This trait makes their potential role for gene therapies clinically safer than other pluripotent stem cells. The use of SSCs for transgenesis in farm animals in comparison to ES cell-based technologies in mice is depicted in Figure 2.

Another alternative for knockout (KO) animal production is nuclear transfer of genetically modified somatic cells, but these cells have limited proliferation capacities, the technique shows inefficient offspring production and postnatal degenerative problems. For these reasons, cloning does not efficiently compete with ES cells or SSC technologies for gene targeting.

Due to their potentials, SSCs have been used to generate KO animals in several species. Transgenic mice generation by using SSCs was first accomplished with the use of retroviral vectors, however with low efficiency[75]. Later on, with the advent of GS cells (SSCs that proliferate *in vitro*) this cell type could be expanded *in vitro* and genetically modified through vectors (viruses or plasmids) with drug resistant genes to allow for the detection of SSCs carrying the transgene. These SSCs were transplanted to recipient mice and took over the spermatogenetic functions in the new environment to ultimately produce transgenic sperm. Transgenic heterozygous males with transgene carrying sperm were mated with females and progeny was produced, near 50% of which carried the transgene[76]. This illustrates the importance of an optimal culture system in order to expand SSC populations and thus increase the efficiency of the transgene incorporation. Soon after, sophisticated techniques added to the gene function modification toolbox such as the CRE/LoxP system for conditional gene targeting, was successfully adapted to the use with SSCs[77]. Interestingly, SSC mediated rat transgenesis was achieved in 2007[78] while parallel efforts with ES cells on this species were only successful until 2010[79]. Just recently, ES cells from domestic animals could be obtained for gene targeting goals[80]. Advances with farm animal SSCs continued in parallel. For instance, goat transgenic

**Table 1 Summary comparison of characteristics of embryonic stem cells and spermatogonial stem cells in the context of transgenesis tecniques**

| Trait | Embryonic stem cells | SSCs |
|---|---|---|
| Mutagenesis technical status | Available for mice. Many genes mutated | Experimental level in mouse[75], rats[78], goats[81], pigs[82] |
| Source | Embryo | Adult testis |
| Age of derivation | Embryonic period | Postnatal period |
| Ethical/legal concerns | Yes | No |
| Efficiency | | 5 × to 10 × higher than with ES cells |
| Cell differentiation state | Undifferentiated - pluripotential | Differentiated up to germ-line |
| Tumorogenesis | Produce teratomas | Do not produce teratomas |
| Chimera formation | (+) | (-) |
| Germline gene transmission | Do not transmit genes from one generation to the next | Transmit genes from one generation to the next |
| *In vitro* phenotype | Colonies with tightly attached cells | GS cells loosely attached (easily dissociated with trypsin)[2] |
| Speed of growth | Faster | Slower |
| Requires other non-transfected SSCs during culture | No | Yes[2] |
| Karyotype | Variable - unstable[1] | Normal - stable |
| Epigenetics | Variable - unstable DNA methylation pattern | Normal - stable DNA methylation pattern |
| Offspring | Abnormal | Normal |

Otherwise specified, information presented is based on[2]: [1]Often lose their germ cell potential due to trisomy[16]; [2]Spermatogonial stem cells (SSCs) that proliferate in culture (GS cells) require specific non-transfected SSC densities during the process of drug selection[118]. ES: Embryonic stem.

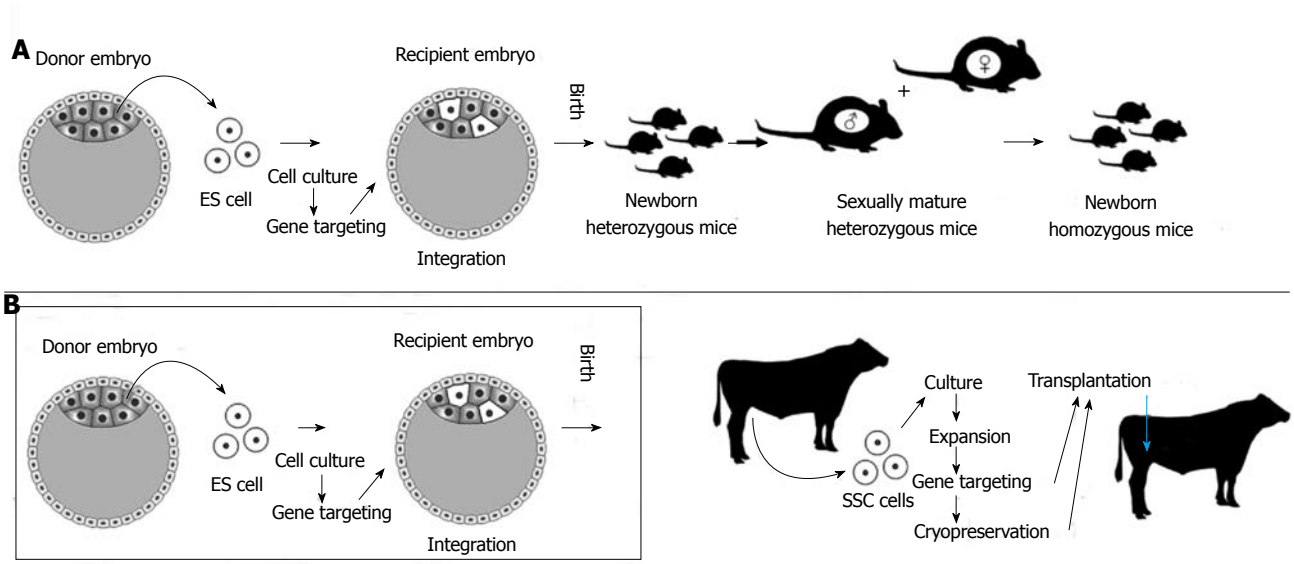


**Figure 2 Alternative ways to exploit stem cells to produce transgenic animals.** A: Standard method (well developed in the mouse): Embryonic stem cells (ES cells) are obtained from a donor mouse blastocyst. These cells are cultured and genetically modified (usually, DNA sequences are inserted on a gene of interest to disrupt its expression or gene knockout). ES cells are injected into another mouse blastocyst so that they can integrate into the embryo (chimera formation) with the hope that some of them will contribute to the male germline. In this case, donor germline cells will undergo homologous recombination (meiosis) in the testis after animals are born and develop towards puberty. The male animals will be heterozygous for the mutated gene and when crossed with their (also heterozygous) sisters, offspring will be obtained homozygous for the transgene; B: Transgenesis technique involving spermatogonial stem cells (SSCs), particularly useful for farm animals, for which ES cell technologies are not feasible. SSCs from postnatal animals can be expanded in culture, subject to gene targeting and transplanted to a recipient foreign testis. This way, steps used with ES cell technologies become redundant (Figure 2B, inside the box). With the advent of spermatogenesis in vitro, even the SSC transplantation procedure will not be necessary. Cryopreservation techniques for SSCs are already available.

sperm for human growth hormone was derived from male goats that had been transplanted with SSCs previously manipulated *in vitro*[81]. In a report from the same group, pig embryos, transgenic for a reporter gene (enhanced green fluorescent protein), could be obtained after oocytes were fertilized with transgenic sperm from culture transduced and later transplanted SSCs[82]. Very recently, bovine gonocytes were transduced through a lentivirus vector and the reporter transgene product proved the success of the procedure[83]. In dogs, a species with a strong potential to provide new models for human genetic diseases, SSCs have been characterized, cultured, genetically modified and allogeneically transplanted with eventual collection of transgenic sperm[84].

## GERM CELL TRANSPLANTATION

SSC transplantation is an important tool for several other biotechnological applications such as gene

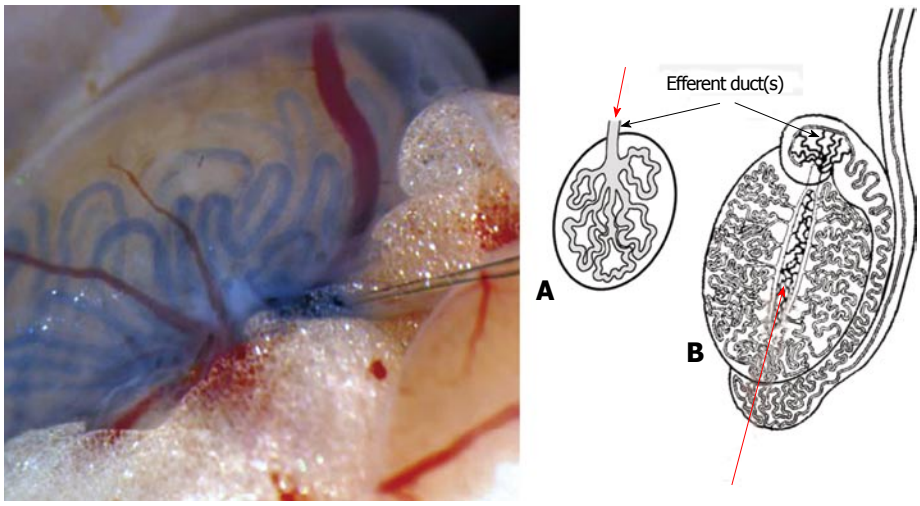


**Figure 3 Species differences on anatomical points for spermatogonial stem cell injection for transplantation.** The transplantation procedure for spermatogonial stem cells (SSCs) is simple in the mouse. In this species, (A) there is a single efferent duct emerging from the rete testis, which is easy to cannulate to inject SSCs (arrow). In most farm animals and men (B) several efferent ducts emerge from the testis, a reason why transplantation of SSCs is often done by injection of SSCs into the rete testis (arrow) with ultrasound guidance.

therapy or fertility restoration. The first SSC successful transplantation was reported in 1994 in the pioneering work of the Brinster's group[85]. Inspired on original hemopoietic fundamental transplantation work[86], this research group isolated germ cells from a donor mice that were later unequivocally identified as SSCs on a recipient mouse testis after injection into the seminiferous tubule system, based on their property to regenerate spermatogenesis in the foreign testicular niche (for a comprehensive review on this technique see[87]). This allogeneic type of transplantation is presently the basis for the functional assay to detect true SSCs in experimental models. The technique could be developed due to the presence of a single, easy to cannulate efferent duct in mouse species and to the fact that the mice strain used was immunocompromised. Unfortunately, this SSC transplantation procedure is technically demanding in species with several efferent ducts because, in these species they tend to be short, thin and highly convoluted (Figure 3). This anatomical condition corresponds to most farm animals[88], primates[89] and humans[90].

Xenogeneic transplantation of SSC cell suspensions into the mouse testis has been attempted by several groups[91]. For the first time, SSCs from a foreign species (rat) not only were able to colonize mouse seminiferous tubules but also to differentiate and produce normal rat sperm[92]. However, attempts with other SSC donor species showed that the farther apart in phylogenetical distance is the donor species from the mouse (recipient species), the lower level of progress of differentiation in spermatogenesis from the transplanted SSCs will be achieved within the foreign seminiferous epithelium environment (for details on SSC transplantation into the mouse testis, involving different donor species, see[91]). Accordingly, domestic animals and human beings as SSC donors are among those species in which this technique is not yet successful. Thus, on these and many other species, functional assays to unequivocally detect the presence of their SSCs are very necessary.

Allogeneic SSC transplantation (SSCs transplanted from a donor animal to a recipient testis of another animal, both from the same species) has proven difficult in species other than rodents, particularly in large animals. Besides anatomical difficulties, each species seems to require specific technical protocols. Allogeneic transplantation has been attempted in several farm animals, including pigs, goats and bulls[93-95]. For instance, transgenic sperm from a donor goat could be unequivocally identified in a recipient buck semen, meaning that transplanted SSCs could colonize and regenerate the spermatogenetic process in the unrelated testis[96]. When the limitations of these techniques are overcome they will facilitate several of the biotechnological applications described on this article. One aspect that still needs to be addressed is the method for depletion of endogenous spermatogenesis in recipient animals. This is typically performed by using either chemical compounds like the alkylating agent busulfan or alternatively testis irradiation. However, protocols need to be species optimized, which requires further research[97,98]. SSC transplantation could also be used for gene delivering purposes in order to increase the frequency of certain genes in animal populations. This would be particularly useful in extensive systems of livestock production, in which animals reproduce through natural service. Surrogate males could be transplanted with SSCs that over-express certain genes linked to increased production traits or from elite bulls in order to disseminate the desired genes in a population. Endangered species could also be benefited with such technologies. For instance, SSCs from the wild species to be preserved could be transferred to a

phylogenetically related domestic animal embryo and this way become incorporated into the germline to eventually produce exotic sperm[99,100], this way saving the endangered germplasm.

As an alternative to SSC transplantation into testicular environments, a new technique was developed in which whole testicular tissue explants (containing SSCs attached to their niche) from the desired species are placed in ectopical places such as the back skin (subcutaneous tissue) of immunocompromised mice. This approach was first successfully tried with mouse testis tissue (donor) grafted into mouse skin (recipient)[101,102]. This technique was designated testis tissue xenografting. When xenotransplanting testicular tissues from prepubertal animals (with only undifferentiated germ cells, including SSCs), complete spermatogenesis developed within the transplanted testicular tissues under the sponsoring of the host (mouse) vascular environment and intrinsic steroidogenesis of the donor tissue. This technique has been so far successful with a wide range of donor species including hamsters, rabbits, dogs, cats, sheep, goats, horses, bovines, alpacas and monkeys[103]. Work has also shown that testicular tissue pieces can be cryopreserved and later used with this technique in various species[104,105].

Tissue xenografting has represented a step forward in SSC biology that circumvents many of the current limitations the transplantation of germ cell suspensions into the seminiferous tubules of foreign recipient animals. This technique has been privileged by researchers during the recent years because of the positive outcomes in a range of very dissimilar species. Recently, a related technique was developed in which no whole tissue pieces are transplanted into the mouse skin but instead an assorted cell suspension composed of germ cells and testicular somatic cells, including Sertoli cells. Interestingly, all these cells reassemble under the mouse skin and testicular tissue becomes organized in a process that has been called *de novo* testicular morphogenesis[106,107]. This novel technique has been tested with testicular cell suspensions from rats[108], mice[109], pigs[110], sheep[111] and recently from a wild pig, the peccary[112]. Testis xenografts have produced so far fertile sperm in pigs and the related peccaries[112-114] demonstrating that SSCs present in the testicular tissues show a correct functioning that leads to the production of viable normal sperm within the body of a foreign species.

## FERTILITY RESTORATION

Infertility is one of the critical side-effects of oncologycal treatments in humans and particularly in children, an effect which is caused either by chemotherapeutic agents or irradiation. A proposed solution for this problem is the restoration of fertility *via* SSC transplantation. Since many of the patients in this situation are prepubertal (*i.e.*, do not yet produce sperm), a current approach consists on banking testicular tissue or cell suspensions prior to the oncological treatment and submitting the patient to an autologous transplantation of the preserved material if fertility problems arise later in life[115] (for recent advances in the methods for preservation of fertility on young male human oncological patients see the comprehensive review from Goossens *et al*[116], 2013). A word of caution is valid regarding safety issues when malignant diseases are managed with transplantation procedures. Invariably, cells suspensions including SSCs to be transplanted should be devoid of cancer cells. In experiments with rats, only as few as 20 leukemia cancer cells were enough to produce the disease when transplanted into healthy rat testis[117]. It is unknown to what extent this is reproducible in humans, but as an obvious measure, cell suspensions should be screened and positively selected for SSCs and/or negatively selected for cancer cells, through approaches such as FACS or special culture conditions[116]. An alternative approach would be to use testis xenografting or allografting with the hope to restore the spermatogenic process ectopically to a degree just enough to produce a few sperm to be later used with ARTs. Nevertheless, although already developed for several animal species (see the topic on Germ cell transplantation above) testicular xenografting requires further investigations to be implemented in humans. In general, more research in monkeys and humans is still needed, particularly on safety issues.

## FINAL REMARKS

The tremendous progress in SSC research during the last decade is undeniable. Appropriate conditions for SSC long-term culture are already widely known for the most important experimental model: the mouse, but research efforts are still being made to develop culture systems suitable for humans and other species, altogether with the study of the very specific SSC nutrient requirements. Advances such as SSC *in vitro* mutagenesis are already possible in the mouse. The imminent unraveling of the molecular mechanisms of SSC pluripotency will bring about many other interesting biotechnological possibilities. The long-awaited accomplishment of SSC transplantation techniques for farm animals and humans is under current development, despite the widespread use of testis xenografting in many species with high rates of success. Finally, spermatogenesis *in vitro* is already possible for laboratory species, so important applications in regenerative medicine and animal reproduction are expected.

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## REFERENCES


1 **Guan K**, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006; **440**: 1199-1203 [PMID: 16565704 DOI: 10.1038/nature04697]

2 **Kanatsu-Shinohara M**, Takehashi M, Shinohara T. Brief history, pitfalls, and prospects of mammalian spermatogonial stem cell research. *Cold Spring Harb Symp Quant Biol* 2008; **73**: 17-23 [PMID: 19022768 DOI: 10.1101/sqb.2008.73.033]

3 **Seandel M**, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP, Rafii S. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature* 2007; **449**: 346-350 [PMID: 17882221 DOI: 10.1038/nature06129]

4 **Yoshida S**, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* 2007; **317**: 1722-1726 [PMID: 17823316 DOI: 10.1126/science.1144885]

5 **Campos-Junior PH**, Costa GM, de Avelar GF, Segatelli TM, Lacerda SM, Aponte PM, de França LR. Morphometric evaluation of the spermatogonial stem cell distribution and niche in vertebrates. *Methods Mol Biol* 2013; **1035**: 35-42 [PMID: 23959980 DOI: 10.1007/978-1-62703-508-8_4]

6 **Hofmann MC**. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol Cell Endocrinol* 2008; **288**: 95-103 [PMID: 18485583 DOI: 10.1016/j.mce.2008.04.012]

7 **Tiptanavattana N**, Thongkittidilok C, Techakumphu M, Tharasanit T. Characterization and in vitro culture of putative spermatogonial stem cells derived from feline testicular tissue. *J Reprod Dev* 2013; **59**: 189-195 [PMID: 23358308]

8 **Lee KH**, Lee R, Lee WY, Kim DH, Chung HJ, Kim JH, Kim NH, Choi SH, Kim JH, Song H. Identification and in vitro derivation of spermatogonia in beagle testis. *PLoS One* 2014; **9**: e109963 [PMID: 25333749 DOI: 10.1371/journal.pone.0109963]

9 **Heidari B**, Rahmati-Ahmadabadi M, Akhondi MM, Zarnani AH, Jeddi-Tehrani M, Shirazi A, Naderi MM, Behzadi B. Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers. *J Assist Reprod Genet* 2012; **29**: 1029-1038 [PMID: 22782689 DOI: 10.1007/s10815-012-9828-5]

10 **Izadyar F**, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, De Rooij DG. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biol Reprod* 2003; **68**: 272-281 [PMID: 12493723]

11 **Nickkholgh B**, Mizrak SC, Korver CM, van Daalen SK, Meissner A, Repping S, van Pelt AM. Enrichment of spermatogonial stem cells from long-term cultured human testicular cells. *Fertil Steril* 2014; **102**: 558-565.e5 [PMID: 24864010 DOI: 10.1016/j.fertnstert.2014.04.022]

12 **Shinohara T**, Avarbock MR, Brinster RL. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 1999; **96**: 5504-5509 [PMID: 10318913]

13 **Shinohara T**, Avarbock MR, Brinster RL. Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. *Dev Biol* 2000; **220**: 401-411 [PMID: 10753526 DOI: 10.1006/dbio.2000.9655]

14 **Orwig KE**, Shinohara T, Avarbock MR, Brinster RL. Functional analysis of stem cells in the adult rat testis. *Biol Reprod* 2002; **66**: 944-949 [PMID: 11906912]

15 **Zheng Y**, Zhang Y, Qu R, He Y, Tian X, Zeng W. Spermatogonial stem cells from domestic animals: progress and prospects. *Reproduction* 2014; **147**: R65-R74 [PMID: 24357661 DOI: 10.1530/REP-13-0466]

16 **Kanatsu-Shinohara M**, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* 2003; **69**: 612-616 [PMID: 12700182 DOI: 10.1095/biolreprod.103.017012]

17 **Shinohara T**, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc Natl Acad Sci U S A* 2000; **97**: 8346-8351 [PMID: 10900001]

18 **van Pelt AM**, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biol Reprod* 1996; **55**: 439-444 [PMID: 8828852]

19 **Lim JJ**, Seol DW, Choi KH, Shin DH, Kim HJ, Song SH, Lee DR. Spermatogonial stem cell enrichment using simple grafting of testis and in vitro cultivation. *Sci Rep* 2014; **4**: 5923 [PMID: 25080919 DOI: 10.1038/srep05923]

20 **Nagano M**, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL. Culture of mouse spermatogonial stem cells. *Tissue Cell* 1998; **30**: 389-397 [PMID: 9787472]

21 **Aponte PM**, Soda T, van de Kant HJ, de Rooij DG. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. *Theriogenology* 2006; **65**: 1828-1847 [PMID: 16321433 DOI: 10.1016/j.theriogenology.2005.10.020]

22 **Aponte PM**, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ, de Rooij DG. Propagation of bovine spermatogonial stem cells in vitro. *Reproduction* 2008; **136**: 543-557 [PMID: 18663014 DOI: 10.1530/REP-07-0419]

23 **Kuijk EW**, Colenbrander B, Roelen BA. The effects of growth factors on in vitro-cultured porcine testicular cells. *Reproduction* 2009; **138**: 721-731 [PMID: 19633132 DOI: 10.1530/REP-09-0138]

24 **Zheng Y**, Tian X, Zhang Y, Qin J, An J, Zeng W. In vitro propagation of male germline stem cells from piglets. *J Assist Reprod Genet* 2013; **30**: 945-952 [PMID: 23779100 DOI: 10.1007/s10815-013-0031-0]

25 **Zhu H**, Liu C, Li M, Sun J, Song W, Hua J. Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSC). *Anim Reprod Sci* 2013; **137**: 45-52 [PMID: 23290695 DOI: 10.1016/j.anireprosci.2012.12.005]

26 **Aponte PM**, van Bragt MP, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. *APMIS* 2005; **113**: 727-742 [PMID: 16480445 DOI: 10.1111/j.1600-0463.2005.apm_302.x]

27 **Nagano MC**. Techniques for culturing spermatogonial stem cells continue to improve. *Biol Reprod* 2011; **84**: 5-6 [PMID: 20944084 DOI: 10.1095/biolreprod.110.088864]

28 **Kanatsu-Shinohara M**, Inoue K, Ogonuki N, Morimoto H, Ogura A, Shinohara T. Serum- and feeder-free culture of mouse germline stem cells. *Biol Reprod* 2011; **84**: 97-105 [PMID: 20844279 DOI: 10.1095/biolreprod.110.086462]

29 **Kanatsu-Shinohara M**, Ogonuki N, Matoba S, Morimoto H, Ogura A, Shinohara T. Improved serum- and feeder-free culture of mouse germline stem cells. *Biol Reprod* 2014; **91**: 88 [PMID: 25210127 DOI: 10.1095/biolreprod.114.122317]

30 **Kokkinaki M**, Djourabtchi A, Golestaneh N. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). *J Stem Cell Res Ther* 2011; **2**: pii: 2488 [PMID: 24466499 DOI: 10.4172/2157-7633.S2-003]

31 **Akhondi MM**, Mohazzab A, Jeddi-Tehrani M, Sadeghi MR, Eidi A, Khodadadi A, Piravar Z. Propagation of human germ stem cells in long-term culture. *Iran J Reprod Med* 2013; **11**: 551-558 [PMID: 24639790]

32 **de Rooij DG**, Griswold MD. Questions about spermatogonia posed and answered since 2000. *J Androl* 2012; **33**: 1085-1095 [PMID: 22879526 DOI: 10.2164/jandrol.112.016832]

33 **Sato T**, Katagiri K, Kubota Y, Ogawa T. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. *Nat Protoc* 2013; **8**: 2098-2104 [PMID: 24091557 DOI: 10.1038/nprot.2013.138]

34 **Yokonishi T**, Sato T, Katagiri K, Ogawa T. In vitro spermatogenesis using an organ culture technique. *Methods Mol Biol* 2013; **927**: 479-488 [PMID: 22992938 DOI: 10.1007/978-1-62703-038-0_41]

35 **Stukenborg JB**, Schlatt S, Simoni M, Yeung CH, Elhija MA, Luetjens CM, Huleihel M, Wistuba J. New horizons for in vitro spermatogenesis? An update on novel three-dimensional culture

systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. *Mol Hum Reprod* 2009; **15**: 521-529 [PMID: 19561342 DOI: 10.1093/molehr/gap052]

36 **Abu Elhija M**, Lunenfeld E, Schlatt S, Huleihel M. Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian J Androl* 2012; **14**: 285-293 [PMID: 22057383 DOI: 10.1038/aja.2011.112]

37 **Tegelenbosch RA**, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res* 1993; **290**: 193-200 [PMID: 7694110]

38 **Kossack N**, Terwort N, Wistuba J, Ehmcke J, Schlatt S, Schöler H, Kliesch S, Gromoll J. A combined approach facilitates the reliable detection of human spermatogonia in vitro. *Hum Reprod* 2013; **28**: 3012-3025 [PMID: 24001715 DOI: 10.1093/humrep/det336]

39 **Oatley JM**, Brinster RL. The germline stem cell niche unit in mammalian testes. *Physiol Rev* 2012; **92**: 577-595 [PMID: 22535892 DOI: 10.1152/physrev.00025.2011]

40 **Johnston DS**, Olivas E, DiCandeloro P, Wright WW. Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. *Biol Reprod* 2011; **85**: 763-769 [PMID: 21653894 DOI: 10.1095/biolreprod.110.087676]

41 **Meng X**, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 2000; **287**: 1489-1493 [PMID: 10688798]

42 **Kubota H**, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 2004; **101**: 16489-16494 [PMID: 15520394 DOI: 10.1073/pnas.0407063101]

43 **Buageaw A**, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. *Biol Reprod* 2005; **73**: 1011-1016 [PMID: 16014811 DOI: 10.1095/biolreprod.105.043810]

44 **Reding SC**, Stepnoski AL, Cloninger EW, Oatley JM. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. *Reproduction* 2010; **139**: 893-903 [PMID: 20154176 DOI: 10.1530/REP-09-0513]

45 **Abbasi H**, Tahmoorespur M, Hosseini SM, Nasiri Z, Bahadorani M, Hajian M, Nasiri MR, Nasr-Esfahani MH. THY1 as a reliable marker for enrichment of undifferentiated spermatogonia in the goat. *Theriogenology* 2013; **80**: 923-932 [PMID: 23987985 DOI: 10.1016/j.theriogenology.2013.07.020]

46 **Zheng Y**, He Y, An J, Qin J, Wang Y, Zhang Y, Tian X, Zeng W. THY1 is a surface marker of porcine gonocytes. *Reprod Fertil Dev* 2014; **26**: 533-539 [PMID: 23683542 DOI: 10.1071/RD13075]

47 **Maki CB**, Pacchiarotti J, Ramos T, Pascual M, Pham J, Kinjo J, Anorve S, Izadyar F. Phenotypic and molecular characterization of spermatogonial stem cells in adult primate testes. *Hum Reprod* 2009; **24**: 1480-1491 [PMID: 19246463 DOI: 10.1093/humrep/dep033]

48 **Altman E**, Yango P, Moustafa R, Smith JF, Klatsky PC, Tran ND. Characterization of human spermatogonial stem cell markers in fetal, pediatric, and adult testicular tissues. *Reproduction* 2014; **148**: 417-427 [PMID: 25030892 DOI: 10.1530/REP-14-0123]

49 **Sachs C**, Robinson BD, Andres Martin L, Webster T, Gilbert M, Lo HY, Rafii S, Ng CK, Seandel M. Evaluation of candidate spermatogonial markers ID4 and GPR125 in testes of adult human cadaveric organ donors. *Andrology* 2014; **2**: 607-614 [PMID: 24902969 DOI: 10.1111/j.2047-2927.2014.00226.x]

50 **He Z**, Kokkinaki M, Jiang J, Dobrinski I, Dym M. Isolation, characterization, and culture of human spermatogonia. *Biol Reprod* 2010; **82**: 363-372 [PMID: 19846602 DOI: 10.1095/biolreprod.109.078550]

51 **Valli H**, Sukhwani M, Dovey SL, Peters KA, Donohue J, Castro CA, Chu T, Marshall GR, Orwig KE. Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. *Fertil Steril* 2014; **102**: 566-580.e7 [PMID: 24890267 DOI: 10.1016/j.fertnstert.2014.04.036]

52 **Aloisio GM**, Nakada Y, Saatcioglu HD, Peña CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH. PAX7 expression defines germline stem cells in the adult testis. *J Clin Invest* 2014; **124**: 3929-3944 [PMID: 25133429 DOI: 10.1172/JCI75943]

53 **Doetschman T**, Williams P, Maeda N. Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev Biol* 1988; **127**: 224-227 [PMID: 3360212]

54 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556]

55 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]

56 **Lee J**, Kanatsu-Shinohara M, Ogonuki N, Miki H, Inoue K, Morimoto T, Morimoto H, Ogura A, Shinohara T. Heritable imprinting defect caused by epigenetic abnormalities in mouse spermatogonial stem cells. *Biol Reprod* 2009; **80**: 518-527 [PMID: 19020300 DOI: 10.1095/biolreprod.108.072330]

57 **de Rooij DG**, Mizrak SC. Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. *Development* 2008; **135**: 2207-2213 [PMID: 18495819 DOI: 10.1242/dev.015453]

58 **Kanatsu-Shinohara M**, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T. Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 2004; **119**: 1001-1012 [PMID: 15620358 DOI: 10.1016/j.cell.2004.11.011]

59 **Kanatsu-Shinohara M**, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Toyokuni S, Shinkai Y, Oshimura M, Ishino F, Ogura A, Shinohara T. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. *Development* 2005; **132**: 4155-4163 [PMID: 16107472 DOI: 10.1242/dev.02004]

60 **Ramirez JM**, Bai Q, Dijon-Grinand M, Assou S, Gerbal-Chaloin S, Hamamah S, De Vos J. Human pluripotent stem cells: from biology to cell therapy. *World J Stem Cells* 2010; **2**: 24-33 [PMID: 21607113 DOI: 10.4252/wjsc.v2.i2.24]

61 **Lee J**, Shinohara T. Epigenetic modifications and self-renewal regulation of mouse germline stem cells. *Cell Res* 2011; **21**: 1164-1171 [PMID: 21747415 DOI: 10.1038/cr.2011.111]

62 **Izadyar F**, Pau F, Marh J, Slepko N, Wang T, Gonzalez R, Ramos T, Howerton K, Sayre C, Silva F. Generation of multipotent cell lines from a distinct population of male germ line stem cells. *Reproduction* 2008; **135**: 771-784 [PMID: 18502893 DOI: 10.1530/REP-07-0479]

63 **Kobayashi T**, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, Sato H, Lee YS, Usui J, Knisely AS, Hirabayashi M, Nakauchi H. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* 2010; **142**: 787-799 [PMID: 20813264 DOI: 10.1016/j.cell.2010.07.039]

64 **Usui J**, Kobayashi T, Yamaguchi T, Knisely AS, Nishinakamura R, Nakauchi H. Generation of kidney from pluripotent stem cells via blastocyst complementation. *Am J Pathol* 2012; **180**: 2417-2426 [PMID: 22507837 DOI: 10.1016/j.ajpath.2012.03.007]

65 **Matsunari H**, Nagashima H, Watanabe M, Umeyama K, Nakano K, Nagaya M, Kobayashi T, Yamaguchi T, Sumazaki R, Herzenberg LA, Nakauchi H. Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs. *Proc Natl Acad Sci USA* 2013; **110**: 4557-4562 [PMID: 23431169 DOI: 10.1073/pnas.1222902110]

66 **Conrad S**, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Bühring HJ, Mattheus U, Mack A, Wagner HJ, Minger S, Matzkies M, Reppel M, Hescheler J, Sievert KD, Stenzl A, Skutella T. Generation of pluripotent stem cells from adult human testis. *Nature* 2008; **456**: 344-349 [PMID: 18849962 DOI: 10.1038/

nature07404]
67 **Tapia N**, Araúzo-Bravo MJ, Ko K, Schöler HR. Concise review: challenging the pluripotency of human testis-derived ESC-like cells. *Stem Cells* 2011; **29**: 1165-1169 [PMID: 21648019 DOI: 10.1002/stem.669]
68 **Golestaneh N**, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI, Dym M. Pluripotent stem cells derived from adult human testes. *Stem Cells Dev* 2009; **18**: 1115-1126 [PMID: 19281326 DOI: 10.1089/scd.2008.0347]
69 **Kossack N**, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. *Stem Cells* 2009; **27**: 138-149 [PMID: 18927477 DOI: 10.1634/stemcells.2008-0439]
70 **Mizrak SC**, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE, Roepers-Gajadien HL, Raya A, Fluiter K, de Reijke TM, de la Rosette JJ, Knegt AC, Belmonte JC, van der Veen F, de Rooij DG, Repping S, van Pelt AM. Embryonic stem cell-like cells derived from adult human testis. *Hum Reprod* 2010; **25**: 158-167 [PMID: 19815622 DOI: 10.1093/humrep/dep354]
71 **Chikhovskaya JV**, Jonker MJ, Meissner A, Breit TM, Repping S, van Pelt AM. Human testis-derived embryonic stem cell-like cells are not pluripotent, but possess potential of mesenchymal progenitors. *Hum Reprod* 2012; **27**: 210-221 [PMID: 22095788 DOI: 10.1093/humrep/der383]
72 **Resnick JL**, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. *Nature* 1992; **359**: 550-551 [PMID: 1383830 DOI: 10.1038/359550a0]
73 **Kimura T**, Tomooka M, Yamano N, Murayama K, Matoba S, Umehara H, Kanai Y, Nakano T. AKT signaling promotes derivation of embryonic germ cells from primordial germ cells. *Development* 2008; **135**: 869-879 [PMID: 18216173 DOI: 10.1242/dev.013474]
74 **Takashima S**, Hirose M, Ogonuki N, Ebisuya M, Inoue K, Kanatsu-Shinohara M, Tanaka T, Nishida E, Ogura A, Shinohara T. Regulation of pluripotency in male germline stem cells by Dmrt1. *Genes Dev* 2013; **27**: 1949-1958 [PMID: 24029916 DOI: 10.1101/gad.220194.113]
75 **Nagano M**, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Transgenic mice produced by retroviral transduction of male germ-line stem cells. *Proc Natl Acad Sci USA* 2001; **98**: 13090-13095 [PMID: 11606778 DOI: 10.1073/pnas.231473498]
76 **Kanatsu-Shinohara M**, Toyokuni S, Shinohara T. Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells. *Biol Reprod* 2005; **72**: 236-240 [PMID: 15456704 DOI: 10.1095/biolreprod.104.035659]
77 **Takehashi M**, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Ogura A, Shinohara T. Adenovirus-mediated gene delivery into mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 2596-2601 [PMID: 17299052 DOI: 10.1073/pnas.0609282104]
78 **Ryu BY**, Orwig KE, Oatley JM, Lin CC, Chang LJ, Avarbock MR, Brinster RL. Efficient generation of transgenic rats through the male germline using lentiviral transduction and transplantation of spermatogonial stem cells. *J Androl* 2007; **28**: 353-360 [PMID: 17108363 DOI: 10.2164/jandrol.106.001511]
79 **Tong C**, Li P, Wu NL, Yan Y, Ying QL. Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. *Nature* 2010; **467**: 211-213 [PMID: 20703227 DOI: 10.1038/nature09368]
80 **Miao X**. Recent advances in the development of new transgenic animal technology. *Cell Mol Life Sci* 2013; **70**: 815-828 [PMID: 22833168 DOI: 10.1007/s00018-012-1081-7]
81 **Zeng W**, Tang L, Bondareva A, Luo J, Megee SO, Modelski M, Blash S, Melican DT, Destrempes MM, Overton SA, Gavin WG, Ayres S, Echelard Y, Dobrinski I. Non-viral transfection of goat germline stem cells by nucleofection results in production of transgenic sperm after germ cell transplantation. *Mol Reprod Dev* 2012; **79**: 255-261 [PMID: 22231935 DOI: 10.1002/mrd.22014]
82 **Zeng W**, Tang L, Bondareva A, Honaramooz A, Tanco V, Dores C, Megee S, Modelski M, Rodriguez-Sosa JR, Paczkowski M, Silva E, Wheeler M, Krisher RL, Dobrinski I. Viral transduction of male germline stem cells results in transgene transmission after germ cell transplantation in pigs. *Biol Reprod* 2013; **88**: 27 [PMID: 23221397 DOI: 10.1095/biolreprod.112.104422]
83 **Kim KJ**, Cho CM, Kim BG, Lee YA, Kim BJ, Kim YH, Kim CG, Schmidt JA, Ryu BY. Lentiviral modification of enriched populations of bovine male gonocytes. *J Anim Sci* 2014; **92**: 106-118 [PMID: 24166994 DOI: 10.2527/jas.2013-6885]
84 **Harkey MA**, Asano A, Zoulas ME, Torok-Storb B, Nagashima J, Travis A. Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: progress toward transgenesis through the male germ-line. *Reproduction* 2013; **146**: 75-90 [PMID: 23690628 DOI: 10.1530/REP-13-0086]
85 **Brinster RL**, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* 1994; **91**: 11298-11302 [PMID: 7972053]
86 **Till JE**, Mcculloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 1961; **14**: 213-222 [PMID: 13776896]
87 **Tang L**, Rodriguez-Sosa JR, Dobrinski I. Germ cell transplantation and testis tissue xenografting in mice. *J Vis Exp* 2012; **(60)**: pii: 3545 [PMID: 22330955 DOI: 10.3791/3545]
88 **Dobrinski I**. Germ cell transplantation and testis tissue xenografting in domestic animals. *Anim Reprod Sci* 2005; **89**: 137-145 [PMID: 16055282 DOI: 10.1016/j.anireprosci.2005.06.020]
89 **Schlatt S**, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E. Germ cell transfer into rat, bovine, monkey and human testes. *Hum Reprod* 1999; **14**: 144-150 [PMID: 10374111]
90 **Faes K**, Tournaye H, Goethals L, Lahoutte T, Hoorens A, Goossens E. Testicular cell transplantation into the human testes. *Fertil Steril* 2013; **100**: 981-988 [PMID: 23850302 DOI: 10.1016/j.fertnstert.2013.06.016]
91 **Honaramooz A**, Yang Y. Recent advances in application of male germ cell transplantation in farm animals. *Vet Med Int* 2010; **2011**: pii: 657860 [PMID: 20953408 DOI: 10.4061/2011/657860]
92 **Clouthier DE**, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. *Nature* 1996; **381**: 418-421 [PMID: 8632797 DOI: 10.1038/381418a0]
93 **Honaramooz A**, Megee SO, Dobrinski I. Germ cell transplantation in pigs. *Biol Reprod* 2002; **66**: 21-28 [PMID: 11751259]
94 **Honaramooz A**, Behboodi E, Blash S, Megee SO, Dobrinski I. Germ cell transplantation in goats. *Mol Reprod Dev* 2003; **64**: 422-428 [PMID: 12589654 DOI: 10.1002/mrd.10205]
95 **Izadyar F**, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD, Woelders H, Kal HB, De Rooij DG. Autologous and homologous transplantation of bovine spermatogonial stem cells. *Reproduction* 2003; **126**: 765-774 [PMID: 14748695]
96 **Honaramooz A**, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. *Biol Reprod* 2003; **69**: 1260-1264 [PMID: 12801978 DOI: 10.1095/biolreprod.103.018788]
97 **Honaramooz A**, Behboodi E, Hausler CL, Blash S, Ayres S, Azuma C, Echelard Y, Dobrinski I. Depletion of endogenous germ cells in male pigs and goats in preparation for germ cell transplantation. *J Androl* 2005; **26**: 698-705 [PMID: 16291964 DOI: 10.2164/jandrol.05032]
98 **Wang DZ**, Zhou XH, Yuan YL, Zheng XM. Optimal dose of busulfan for depleting testicular germ cells of recipient mice before spermatogonial transplantation. *Asian J Androl* 2010; **12**: 263-270 [PMID: 20010847 DOI: 10.1038/aja.2009.67]
99 **Lacerda SM**, Costa GM, Campos-Junior PH, Segatelli TM, Yazawa R, Takeuchi Y, Morita T, Yoshizaki G, França LR. Germ cell transplantation as a potential biotechnological approach to fish reproduction. *Fish Physiol Biochem* 2013; **39**: 3-11 [PMID: 22290474 DOI: 10.1007/s10695-012-9606-4]
100 **Roe M**, McDonald N, Durrant B, Jensen T. Xenogeneic transfer of adult quail (Coturnix coturnix) spermatogonial stem cells to embryonic chicken (Gallus gallus) hosts: a model for avian

conservation. *Biol Reprod* 2013; **88**: 129 [PMID: 23575150 DOI: 10.1095/biolreprod.112.105189]

101 **Honaramooz A**, Snedaker A, Boiani M, Schöler H, Dobrinski I, Schlatt S. Sperm from neonatal mammalian testes grafted in mice. *Nature* 2002; **418**: 778-781 [PMID: 12181567 DOI: 10.1038/nature00918]

102 **Schlatt S**, Honaramooz A, Boiani M, Schöler HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. *Biol Reprod* 2003; **68**: 2331-2335 [PMID: 12606381 DOI: 10.1095/biolreprod.102.014894]

103 **Arregui L**, Dobrinski I. Xenografting of testicular tissue pieces: 12 years of an in vivo spermatogenesis system. *Reproduction* 2014; **148**: R71-R84 [PMID: 25150043 DOI: 10.1530/REP-14-0249]

104 **Jahnukainen K**, Ehmcke J, Hergenrother SD, Schlatt S. Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. *Hum Reprod* 2007; **22**: 1060-1067 [PMID: 17166865 DOI: 10.1093/humrep/del471]

105 **Zeng W**, Snedaker AK, Megee S, Rathi R, Chen F, Honaramooz A, Dobrinski I. Preservation and transplantation of porcine testis tissue. *Reprod Fertil Dev* 2009; **21**: 489-497 [PMID: 19261226]

106 **Dobrinski I**. De novo morphogenesis of functional testis tissue after ectopic transplantation of isolated cells. *Organogenesis* 2007; **3**: 79-82 [PMID: 19279705]

107 **Dores C**, Dobrinski I. De novo morphogenesis of testis tissue: an improved bioassay to investigate the role of VEGF165 during testis formation. *Reproduction* 2014; **148**: 109-117 [PMID: 24803491 DOI: 10.1530/REP-13-0303]

108 **Gassei K**, Schlatt S, Ehmcke J. De novo morphogenesis of seminiferous tubules from dissociated immature rat testicular cells in xenografts. *J Androl* 2006; **27**: 611-618 [PMID: 16582408 DOI: 10.2164/jandrol.05207]

109 **Kita K**, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, Nagashima Y, Aoki I, Taniguchi H, Noce T, Inoue K, Miki H, Ogonuki N, Tanaka H, Ogura A, Ogawa T. Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. *Biol Reprod* 2007; **76**: 211-217 [PMID: 17065598 DOI: 10.1095/biolreprod.106.056895]

110 **Honaramooz A**, Megee SO, Rathi R, Dobrinski I. Building a testis: formation of functional testis tissue after transplantation of isolated porcine (Sus scrofa) testis cells. *Biol Reprod* 2007; **76**: 43-47 [PMID: 17005941 DOI: 10.1095/biolreprod.106.054999]

111 **Arregui L**, Rathi R, Megee SO, Honaramooz A, Gomendio M, Roldan ER, Dobrinski I. Xenografting of sheep testis tissue and isolated cells as a model for preservation of genetic material from endangered ungulates. *Reproduction* 2008; **136**: 85-93 [PMID: 18390693 DOI: 10.1530/REP-07-0433]

112 **Campos-Junior PH**, Costa GM, Avelar GF, Lacerda SM, da Costa NN, Ohashi OM, Miranda Mdos S, Barcelos LS, Jorge EC, Guimarães DA, de França LR. Derivation of sperm from xenografted testis cells and tissues of the peccary (Tayassu tajacu). *Reproduction* 2014; **147**: 291-299 [PMID: 24324205 DOI: 10.1530/REP-13-0581]

113 **Honaramooz A**, Cui XS, Kim NH, Dobrinski I. Porcine embryos produced after intracytoplasmic sperm injection using xenogeneic pig sperm from neonatal testis tissue grafted in mice. *Reprod Fertil Dev* 2008; **20**: 802-807 [PMID: 18842182]

114 **Nakai M**, Kaneko H, Somfai T, Maedomari N, Ozawa M, Noguchi J, Ito J, Kashiwazaki N, Kikuchi K. Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts. *Reproduction* 2010; **139**: 331-335 [PMID: 20015869 DOI: 10.1530/REP-09-0509]

115 **Struijk RB**, Mulder CL, van der Veen F, van Pelt AM, Repping S. Restoring fertility in sterile childhood cancer survivors by autotransplanting spermatogonial stem cells: are we there yet? *Biomed Res Int* 2013; **2013**: 903142 [PMID: 23509797 DOI: 10.1155/2013/903142]

116 **Goossens E**, Van Saen D, Tournaye H. Spermatogonial stem cell preservation and transplantation: from research to clinic. *Hum Reprod* 2013; **28**: 897-907 [PMID: 23427228 DOI: 10.1093/humrep/det039]

117 **Jahnukainen K**, Hou M, Petersen C, Setchell B, Söder O. Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. *Cancer Res* 2001; **61**: 706-710 [PMID: 11212272]

118 **Takehashi M**, Kanatsu-Shinohara M, Shinohara T. Generation of genetically modified animals using spermatogonial stem cells. *Dev Growth Differ* 2010; **52**: 303-310 [PMID: 20148923 DOI: 10.1111/j.1440-169X.2009.01167.x]

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REVIEW

# Osteogenic differentiation of amniotic fluid mesenchymal stromal cells and their bone regeneration potential

Caterina Pipino, Assunta Pandolfi

Caterina Pipino, Assunta Pandolfi, Department of Medical, Oral and Biotechnological Sciences, "G. d'Annunzio" University, 66013 Chieti, Italy
Caterina Pipino, Assunta Pandolfi, Aging Research Center, Ce.S.I., "Gabriele d'Annunzio" University Foundation, StemTeCh Group, 66013 Chieti, Italy
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Correspondence to: Caterina Pipino, PhD, Aging Research Center, Ce.S.I., "Gabriele d'Annunzio" University Foundation, Room 421, Via Luigi Polacchi, 11, 66013 Chieti, Italy. c.pipino@unich.it
Telephone: +39-0871-541424
Fax: +39-0871-541425
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## Abstract

In orthopedics, tissue engineering approach using stem cells is a valid line of treatment for patients with bone defects. In this context, mesenchymal stromal cells of various origins have been extensively studied and continue to be a matter of debate. Although mesenchymal stromal cells from bone marrow are already clinically applied, recent evidence suggests that one may use mesenchymal stromal cells from extra-embryonic tissues, such as amniotic fluid, as an innovative and advantageous resource for bone regeneration. The use of cells from amniotic fluid does not raise ethical problems and provides a sufficient number of cells without invasive procedures. Furthermore, they do not develop into teratomas when transplanted, a consequence observed with pluripotent stem cells. In addition, their multipotent differentiation ability, low immunogenicity, and anti-inflammatory properties make them ideal candidates for bone regenerative medicine. We here present an overview of the features of amniotic fluid mesenchymal stromal cells and their potential in the osteogenic differentiation process. We have examined the papers actually available on this regard, with particular interest in the strategies applied to improve *in vitro* osteogenesis. Importantly, a detailed understanding of the behavior of amniotic fluid mesenchymal stromal cells and their osteogenic ability is desirable considering a feasible application in bone regenerative medicine.

**Key words:** Mesenchymal stromal cells; Amniotic fluid; Amniotic fluid mesenchymal stromal cells; Amniotic fluid stem cells; Osteogenesis; Bone regeneration

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**Core tip:** Several papers regarding the osteogenic differentiation potential of cells isolated from amniotic fluid have been published so far with particular attention to various feasible approaches to improving differentiation both *in vitro* and *in vivo*. Hence, an overview is necessary on the data reported up to now in order to understand the potential of amniotic fluid-derived cells in bone regenerative medicine. This review takes a general look at the current state-of-the-art of the osteogenic ability of amniotic fluid-derived cells and the different strategies available to improve bone regeneration.

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## INTRODUCTION

In the last few decades degenerative lesions of the musculoskeletal system have become increasingly common, leading to social and economic problems, and intensifying the demand on medical services[1]. Common examples are temporary and permanent disability due to osteoarthritis or fractures caused by loss of bone mass such as osteoporosis or osteopenia, particularly in the elderly. In the younger population widespread active lifestyles including sport activities have increased the possibility of bone and cartilage damage. Moreover, bone defects mostly emerge from trauma, tumor resection and congenital malformation. So far, the most effective clinical method for the reconstruction of large bone defects is the use of autogenous bone grafts, commonly harvested from the posterior iliac crest and transplanted into a local bone defect[2]. The weakness of this method is the surgical stress the patient undergoes in extracting bone with possible subsequent inflammation and donor site morbidity. Furthermore, the quantity of extractable bone is limited.

Recently, advances in the field of stem cells have come to represent a valid alternative to this method[3]. Nowadays there is growing interest in therapies based on mesenchymal stromal cells (MSCs) as a potential effective treatment for bone defects. MSCs are multipotent cells with the potential to engender a range of specialized cell types, such as osteoblasts, chondrocytes and adipocytes[4]. The mesenchymal progenitor cells in the bone marrow are able to differentiate into osteoblasts following the influence of multiple osteogenic signals[5,6]. In particular, after fracture MSCs of bone marrow are transferred to the site of bone injury through peripheral blood, thus enhancing the healing potential of local MSCs[7]. In this condition, bone morphogenic proteins (BMPs) play an important role. Indeed, osteoblastic differentiation begins when the BMPs bind their receptors activating the transcription factors runt-related transcription factor 2 (Runx2) and Osterix, and subsequent downstream osteoblast specific genes, through activation of a Wnt/LRP5 cascade, which is crucial in bone mass modeling[5,8,9].

Regarding the principal signaling pathways involved in MSC differentiation, various evidences have suggested that during cultivation *in vitro,* an inverse relationship exists between the commitment of MSCs toward osteogenic and adipogenic lineages[10]. Several cell signaling cascades are involved in this cell fate decision between osteo- and adipogenesis[11]. The master regulator of osteogenesis is Runx2, the gene target of many signaling pathways, including but not limited to transforming growth factor-beta 1 (TGF-β1), BMP[12], Wingless type (Wnt)[13], Hedgehog (HH)[14], and (NEL)-like protein type 1 (NELL-1)[15].

Thus, considering the crucial role of MSCs in bone healing, the strategy of using the osteogenic potential of such cells transplanted into the bone defect seems promising[7,16].

Mesenchymal stromal cells derived from bone marrow (BM-MSCs) have been used in clinical trials for the treatment of bone defects[17]. However, bone marrow aspiration is a difficult, invasive and painful procedure for the donor. The amount of BM-MSCs is usually between 0.001% and 0.01% of the total population and, in addition, the BM-MSC number and differentiation potential may be affected by increasing donor age. This implies an extensive *in vitro* expansion of such cells before transplantation, thus increasing the risk of possible differentiation induction and epigenetic modifications[18]. On the other hand, the use of allogeneic BM-MSCs for bone repair is unsuitable due to immune rejection[19].

As an alternative, mesenchymal stromal cells can be found in various adult organs and tissues[20], including blood[21], adipose tissue[22] and dermis[23]. Although sharing similar properties, these MSCs from various sources have different gene expression profile and differentiation ability[24].

Mesenchymal stem-like cells have also been recognized in fetal blood, liver and bone marrow[25]. In this regard, Guillot and colleagues have shown that first trimester fetal blood, liver, and bone marrow MSCs possess greater osteogenic differentiation potential than adult BM-MSCs[26]. They found through quantitative real-time RT-PCR that 16 osteogenic specific genes (*OC*, *ON*, *BSP*, *OP*, *Col1*, *PCE*, *Met2A OPG*, *PHOS1*, *SORT*, *ALP*, *BMP2*, *CBFA1*, *OSX*, *NOG*, *IGFⅡ*) were more expressed in fetal MSCs under basal conditions and were up-regulated during osteogenic differentiation both *in vitro* and *in vivo*[26]. These cells are more primitive than adult MSCs[25], therefore potentially promising for therapeutic use in regenerative medicine, even if isolating them is subject to considerable public unease.

An alternative approach could result from the use of MSCs derived from extra-embryonic tissues, which possess the advantage of being isolated from tissues normally discarded after birth, hence exempt from ethical concern, such as amniotic fluid, umbilical cord and placenta[27-29].

This review will focus on the biological properties of MSCs isolated from amniotic fluid (AF-MSCs) with particular attention to their *in vitro* osteogenic differentiation potential with a view to possible final application in bone regenerative medicine (Figure 1). Of note, these cells possess a greater proliferative capacity, lower immunological reactivity and lower risk of graft-versus-host disease than those derived from adult bone marrow[30]. Importantly, compared with other MSC sources, such as umbilical cord blood-derived MSCs, AF-MSCs are more easily isolated and show better proliferation ability[31].

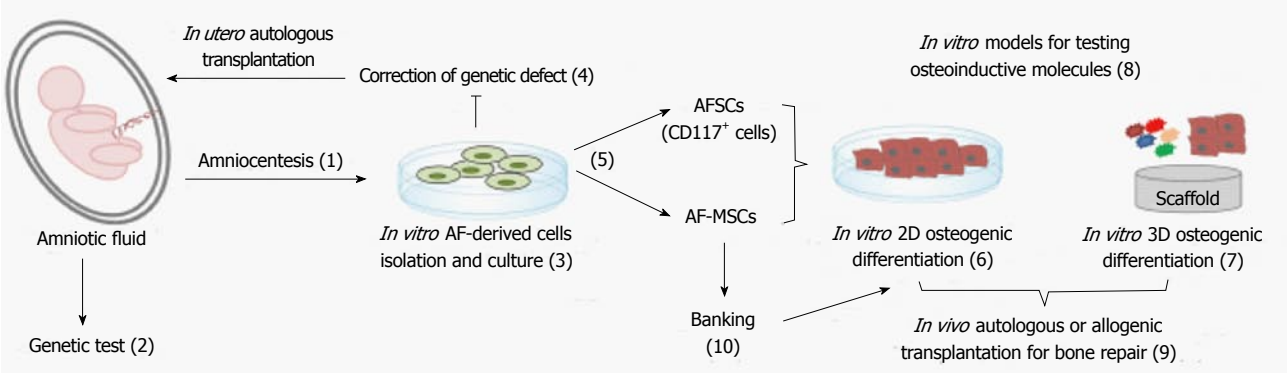


**Figure 1 *In vitro* and *in vivo* application of amniotic fluid-derived cells.** Amniotic fluid-derived cells isolated from amniotic fluid samples obtained following ultrasound-guided amniocentesis (1) for genetic test (2), could be *in vitro* expanded (3). These cells may be genetically manipulated and transplanted back into the same fetus (4). AF-derived cells can be used unselected (AF-MSCs) or selected for CD117 (AFSCs) (5). Both cellular populations can be *in vitro* differentiated in 2D (6) or 3D (7) culture conditions. They represent an ideal *in vitro* model for testing osteoinductive molecules (8) and for *in vivo* autologous or allogeneic transplantation (9). Alternatively, they could also be banked (10) and adopted post-thawing.

In particular, AF-MSCs are autologous to the foetus representing an attractive source for the treatment of perinatal disorders such as congenital malformations[32,33]. Hence, affected children could benefit from their own cells which could be banked, expanded in culture or properly engineered and implanted in the neonatal period. In addition, AF-MSCs are semi-allogeneic to each parent, therefore potentially useful for the other members of the family[34].

Moreover, the beneficial effect of AF-MSCs observed in preclinical studies, such as lung injury[35], ischemic heart[36], acute bladder injury[37], neovascularization[38], encourages their future application in regenerative medicine tissue engineering.

## AMNIOTIC FLUID-DERIVED CELLS

Human amniotic fluid, contained within the amniotic cavity, begins to appear at the second week of gestation. It surrounds the growing fetus, protects from outside injury and acts as the vehicle with the mother[30]. It contains a variety of cells arising from all three germ layers (ectoderm, mesoderm and endoderm) routinely obtained for prenatal diagnosis of fetal abnormalities during second trimester pregnancy, through a minimally invasive technique. Amniotic fluid cellular composition substantially depends on the gestational age[27]. Most of the cells present in the amniotic fluid are terminally differentiated and have low proliferative capacity[32]. Three major cellular types can be classified based on morphological, biochemical and growth characteristics: epithelioid, amniotic fluid and fibroblastic cells[39].

Interestingly, a population that expresses the surface antigen c-kit (CD117), stem cell factor receptor, can be isolated from amniotic fluid. These cells, named amniotic fluid stem cells (AFSCs), represent about 1% of the total cells[40]. It should be noted that, mesenchymal stromal cells (AF-MSCs) with a multilineage differentiation potential are present in the amniotic fluid[41,42].

AF-MSCs are highly proliferative with a normal karyotype after long-term *in vitro* culture and do not form teratomas when transplanted *in vivo*[43]. They do not display any hematopoietic molecular markers (CD14, CD31 and CD45), while they express the intracellular stemness markers TERT, SOX2 and Nanog and the surface adhesion molecules CD29, CD58, CD166 and CD90[44].

The absence of HLA-DR and presence of HLA-ABC suggest that these cells may be applicable in immune-mediated disorders as well as in the treatment of graft-versus-host disease[43]. It is also important that AF-MSCs display a broad differentiation potential toward multiple lineages (*i.e.,* adipogenic, chondrogenic, myogenic and osteogenic)[34]. They possess an intermediate differentiation potential between embryonic (pluripotent) and adult cells with advantages over both. Compared to adult cells, AF-MSCs possess greater differentiation potential and more primitive properties with fewer accumulated mutations[45]. Respect to embryonic stem cells (ESCs), AF-MSCs possess the advantage to do not form teratomas when transplanted *in vivo.* Indeed, although ESCs are pluripotent cells which maintain high plasticity and extensive self-renewal capacity, possible host immune rejection after allotransplantation and the formation of tumors when injected undifferentiated or partially differentiated *in vivo*, raise safety concerns[46-48]. Furthermore, their clinical use is limited by ethical issues due to the need to isolate them from the inner cell mass of a blastocyst[49].

In the last few years methods able to generate patient-specific pluripotent stem cells (iPSCs) from adult cells have been developed to overcome the limitations associated with ESCs[50,51]. Such iPSCs, obtained through the ectopic expression of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc), are molecularly and functionally similar to ESCs. They show similar morphology and growth properties, express pluripotency markers, are able to generate germline-competent chimeras and form tumors when injected into immune-compromised mice[51]. Although iPSCs

allow one to overcome both the ethical and the tissue compatibility problems of ESCs, current studies are still investigating the safety profile of these cells for therapeutic application[52]. The main limitation related to iPSC generation is the use of retroviruses or lentiviruses, which could cause mutagenesis leading to a risk for teratogenesis.

Therefore, given the easier accessibility and the faster availability of a great number of AF-MSCs in culture in comparison to iPSCs, AF-MSCs may hold great promise in regenerative medicine.

However, since iPSCs seem one of the most promising future sources of stem cells for tissue regeneration, research is going ahead to find the best cell source to reprogram and develop alternative methods for generating pluripotent cells using non integrating systems. In this context, recent evidences has suggested that cells derived from amniotic fluid are more easily and rapidly reprogrammed than adult cells[53-55]. More recently, iPSCs have been developed from amniotic fluid cells without ectopic factors by culture in an appropriate medium[56], thus reinforcing their potential application in cellular replacement therapies.

These important features, together with the high proliferation rate, ease of retrieval and more stable profile, provide a convincing proof-of-principle for potential autologous application of AF-MSCs for bone regeneration in perinatal applications[57]. Furthermore, a bank of such cells is achievable and may in the future provide a plentiful source for autologous therapy in adulthood as well as for transplantation into HLA-matched recipients.

## *IN VITRO* OSTEOGENIC DIFFERENTIATION

The osteogenic differentiation capacity of AF-derived cells obtained from various sources (human, sheep, mouse and rat) has been broadly documented[39,40,58,59]. Some studies have reported the great potential of c-kit selected amniotic fluid cells prior to osteogenic differentiation[40], while others have demonstrated that unselected cells are also able to properly differentiate into osteo-specific cells[13,41,44,60]. In this review we mainly discuss the *in vitro* osteogenic differentiation of AF-MSCs with occasional mention of papers in which authors use c-kit selected cells, named AFSCs (Table 1). It should be pointed out that, compared to BM-MSCs, AF-MSCs have higher self-renewal capacity and are more potent for lineage-specific differentiation[40]. Therefore, considering also their more easily isolation and the great number of available cells, AF-MSCs may represent excellent candidates for cell replacement therapies[61]. Of note, even if there are other MSC sources easily available, like adipose-derived MSCs, the osteogenic capacity of such cells compared to BM-MSCs is now still debated[62].

The conventional protocol applied to induce osteogenic differentiation of AF-MSCs consists of basal medium, such as Dulbecco's modified Eagle's medium, supplemented with 10% Fetal Bovine Serum, β-glycerolphosphate, ascorbic acid and dexamethasone (standard osteogenic medium). For the purpose of assessing *in vitro* differentiation, protein and gene expression of specific osteogenic markers [*e.g.*, alkaline phosphates (ALP), Collagen type I, bone sialoprotein, osteocalcin, RUNX2] are usually evaluated in addition to specific colorimetric assays, such as ALP, Alizarin Red S and Von Kossa staining.

However, various approaches have been applied to improve and accelerate differentiation of amniotic fluid-derived cells into osteogenic cells. Attempts have been made to produce osteoblastic cells from amniotic fluid samples in a very short time, using a single step culture procedure, which allows a 20-d reduction in culture time[60].

One notes that several studies have demonstrated how such osteoinductive molecules are able to improve the osteogenic differentiation process[44,63-68].

In detail, then, AF-MSCs transfected with a defective adenoviral vector expressing human lim mineralization protein 3, an intracellular positive regulator of osteoblast differentiation, showed downregulation of stemness kruppel-like factor-4 and then increased osteogenic differentiation[63].

Again, Simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor able to act on cholesterol endogenous synthesis, has been shown to be efficient in stimulating new bone formation. When added to osteogenic differentiation medium, Simvastatin was able to induce massive osteogenic differentiation of AF-MSCs, as observed by Alizarin Red S staining, and increased expression of typical osteogenic genes, such as osteopontin (OPN) and osteocalcin[64].

Herbal medicines have also been used to improve the osteogenic differentiation of AF-MSCs. Naringin, the main active compound of Chinese herbal medicine (Rhizoma drynariae), proved able to enhance osteogenic differentiation, increasing ALP activity and the expression of the osteogenic specific markers OPN, Collagen I, bone morphogenetic protein 4 (BMP4), RUNX2, β-catenin and cyclin D1[66].

Another traditional Chinese herbal medicine commonly used in treating orthopedic disorders, Curculigoside, was found to have a positive effect on the osteogenic differentiation of human AFSCs[65].

Moreover, during osteogenic differentiation of both amniotic fluid and dental pulp stem cells, the *in vitro* addition of Ferutinin, a phytoestrogen able to prevent osteoporosis caused by ovariectomy-induced estrogen deficiency, was efficacious in enhancing the production of a calcified matrix[67].

It has been noted that, in co-culture with osteoblasts obtained from the differentiation of dental pulp stem cells AF-MSCs prove able to generate osteoblasts. The same effect was observed when AF-MSCs were

**Table 1 Studies describing *in vitro* modification of standard culture condition to induce osteogenic differentiation of AF-derived cells**

| Cell source | Methods to induce osteogenic differentiation | Ref. |
|---|---|---|
| hAF-MSCs | Culture in standard osteogenic medium on sandblasted and acid etching titanium (SLA titanium) | [60] |
| hAF-MSCs | Transfection with a defective adenoviral vector expressing human LMP3 | [63] |
| hAFSCs | Standard osteogenic medium plus curculigoside | [65] |
| hAFSCs | Standard osteogenic medium plus naringin | [66] |
| hAFSCs | 100 μmol/L 2P-ascorbic acid, 100 nmol/L dexamethasone, supplemented with different concentrations of Ferutinin | [67] |
| hAF-MSCs | Induction of miR-21 | [71] |
| hAFSCs | Medium containing 50 nmol/L rhBMP-7, 50 mg/mL ascorbic acid, and 10 mmol/L b-glycerophosphate on nanofibrous or solid walled scaffolds | [86] |
| hAFSCs | 10 nmol/L dexamethasone, 6 mmol/L β-glycerol phosphate, 50 mg/mL ascorbic acid 2-phosphate, 50 ng/mL L-thyroxine on electrospun nanofiber meshes | [88] |
| hAFSCs | Standard osteogenic medium on:<br>(1) Fibroin scaffold;<br>(2) Collagen scaffold;<br>and (3) Poly-D,L-lactic acid scaffold; | [89] |
| hAFSCs | Standard osteogenic medium on microfibrous starch and poly(ε-caprolactone) scaffold | [90] |
| hAFSCs | Medical-grade poly-ε-caprolactone scaffold | [91] |
| hAFSCs | Standard osteogenic medium on collagen matrix derived from porcine bladder submucosa matrix and poly (lactide-co-glycolide) | [92] |
| hAFSCs | Standard osteogenic medium on construct composed of collagen type I | [93] |
| hAFSCs | α-MEM plus 17% FBS, 1 μmol/L dexamethasone, 6 mmol/L of β-glycerol phosphate, 50 μg/mL ascorbic acid 2-phosphate, and 50 ng/mL thyroxine on biomaterial named Skelite | [38] |

MSCs: Mesenchymal stromal cells; AF-MSCs: MSCs isolated from amniotic fluid; AFSCs: Amniotic fluid stem cells; LMP3: Lim mineralization protein 3.

cultured in the conditioned medium of osteoblasts-differentiated dental pulp stem cells[69].

Moreover, some findings show that microRNAs (miRNAs) are involved in determining the fate of stem cells[70]. For example, a recent paper has shown the role of miR-21 in accelerating osteogenesis of AF-MSCs[71]. In that work two cellular populations were isolated from amniotic fluid: spindle-shaped (SS) and round-shaped (RS). Interestingly, induction of miR-21 was found to accelerate osteogenesis more in the SS population than in RS cells[71].

Finally, human AF-MSCs analyzed by an atomic force microscope during osteogenic differentiation showed a decrease in cell elasticity, which is typical of mature osteoblasts; thus the mechanical properties of AF-MSCs again add to the interest in applying them in bone regenerative medicine[72].

Up to now, little is known about the cues regulating the AF-MSCs' ability to differentiate to osteoblasts. In depth study of the mechanism involved in the osteogenesis of AF-MSCs may hence help to develop standard protocols for clinical application in bone regenerative medicine. In this context, there is some evidence to suggest a role by the canonical Wnt signal pathway in bone formation as activation of this pathway stimulates osteoprogenitor proliferation and osteogenesis of human MSCs[73,74]. Wnt signaling is also involved in AF-MSC commitment toward osteogenesis[13].

Recently, we identified for the first time, the presence of Calcium Sensing Receptor (CaSR) in ovine and human AF-MSCs[44,68]. CaSR, originally cloned from parathyroid glands, acts by controlling the secretion of parathyroid hormone in response to changes in extracellular calcium levels[75]. However, it is well known that CaSR plays an important role in controlling osteoblasts as well as in osteoclast recruitment, differentiation and survival *via* multiple intracellular signals[76]. Interestingly, we observed that CaSR expression in both ovine and human AF-MSCs increased at the membrane when cells were treated with calcimimetic R-568, a molecule able to modulate bone cell metabolism *via* CaSR[77]. This effect was abolished by CaSR allosteric inhibitor Calhex-231 and by selective inhibitor NPS-2143. Importantly, downregulation of CaSR by a gene-silencing approach confirmed the crucial role of CaSR in supporting osteogenic differentiation[44].

These findings support the role of calcimimetics in the osteogenic differentiation of AF-MSCs, and suggest a strategy to develop therapy against bone injury.

Although the aforementioned studies have shown that AF-MSCs can be made to differentiate into osteoblasts *in vitro*[40,41] and some pathways have been investigated, more studies and clinical trials are needed before AF-MSCs can be applied clinically for *in vivo* bone regeneration.

## TISSUE ENGINEERING APPROACHES FOR *IN VIVO* BONE REGENERATION

For a long period time autogenous bone grafting was considered the gold standard for *in vivo* bone regeneration[78]. Because of its limited availability and potential donor site morbidity, several bone substitutes have been successfully tried as an alternative, combining principles of biology and engineering. *In vivo* bone regeneration based on tissue engineering using scaffolds offers a plausible way of creating a favorable microenvironment for cells[79].

The choice of an appropriate scaffold along with selection of the best suited cell source is currently

**Table 2 Studies describing *in vivo* osteogenic differentiation of AF-derived cells**

| Cell source | Scaffold | Animal model | Ref. |
|---|---|---|---|
| oAF-MSCs | Magnesium-enriched hydroxyapatite/collagen-based scaffold | Sheep model of sinus augumentation | [85] |
| hAFSCs | Nanofibrous or solid walled scaffolds | Midsagittal incision made on the dorsa and two subcutaneous pockets created using blunt dissection on male outbred thymic nude mouse model (nu/nu) | [86] |
| hAFSCs | Fibroin scaffolds compared to collagen and poly-D,L-lactic acid scaffolds | Dorsal mid-sagittal incision made on the dorsa and two subcutaneous pockets were created using blunt dissection male outbred rats | [89] |
| hAFSCs | Microfibrous starch and poly(ε-caprolactone) scaffold | Subcutaneous implantation into the dorsal side of athymic female nude rats | [91] |
| hAFSCs | Construct composed of collagen type I | Critical-sized rat calvarial defect after oral administration of phytoestrogen ferutinin | [93] |
| hAFSCs | Biomaterial Skelite | Subcutaneous implantation in the back of CD-1 nu/nu mice | [38] |

MSCs: Mesenchymal stromal cells; AF-MSCs: MSCs isolated from amniotic fluid; AFSCs: Amniotic fluid stem cells.

being widely studied.

As discussed above, the advantages of using amniotic fluid-derived cells are well established. Their high proliferation and osteogenic differentiation ability, together with the possibility of autologous or non-immunogenic transplantation and the absence of teratoma formation, makes possible a three-dimensional application of amniotic fluid cells in bone regeneration.

In general, a scaffold is a three-dimensional construct able to support adhesion, proliferation, and function of appropriate cells[78]. Stimuli mimicking the *in vivo* bone environment are needed for tissue-engineered constructs. It is well known that the bone regeneration ability of MSCs is mainly due to their paracrine effects[80]. They secrete bioactive substances that are able to enhance migration, proliferation, and differentiation of the neighboring resident cells[81,82]. Thus, what is required is the right combination of cells and the most appropriate support and factors.

Various synthetic and non-synthetic scaffolds have been employed to support osteogenic differentiation of AF-MSCs. One commonly used non-synthetic scaffold is collagens, which are present in the bone tissue, where they stimulate MSCs to differentiate into osteoblasts, initiating new bone formation[78]. This natural scaffolds are frequently used for their high availability, biological plasticity, biocompatibility, biodegradability and non-toxicity[83,84].

It has been found that osteoblasts obtained from AF-MSCs were able to adhere and grow well on SLA (Sandblasted and Acid Etching) titanium surfaces, materials commonly utilized in dental implantology, as revealed by electron microscopy observation[60].

Berardinelli *et al*[85] have demonstrated that a biomimetic commercial scaffold (magnesium-enriched hydroxyapatite (MgHA)/collagen-based scaffold) engineered with ovine AF-MSCs improves bone regeneration in a sheep model of sinus augmentation. Of note, the surface of this commercial scaffold was able to entrap a very high concentration of cells ($1 \times 10^7$ cells/cm$^2$) under dynamic cultural conditions. The osteoinductive properties of this scaffold, together with the potential of ovine AF-MSCs clearly accelerated the formation of new bone.

Human AFSCs seeded on nanofibrous (NF) or solid walled (SW) scaffolds were induced in rhBMP-7-containing medium for 7 d and implanted into male outbred thymic nude mice (nu/nu). Six weeks after implantation, bone formation was found on scaffolds as noted by von Kossa staining with greater mineralization on NF than SW scaffolds[86].

Synthetic NF scaffolds were developed with a morphology similar to that of natural collagen fibers, the aim being to mimic the morphological function of collagen fibers[87].

Human AFSCs were differentiated on electrospun nanofiber meshes and compared to BM-MSCs. In these experimental conditions, the cells displayed a delay in alkaline phosphatase activity, but elevated mineral deposition after 4 wk in culture, compared to BM-MSCs[88]. The nanofiber mesh scaffold possesses high porosity, large surface-area-to-volume ratios and size scale similar to extracellular matrix components. It allows the attachment of the cells and act as an efficient vehicle to deliver them to a defective site. A study by Maraldi *et al*[89] demonstrated that mineralization of hAFSCs tended to be enhanced on fibroin scaffolds, better than collagen and poly-D,L-lactic acid scaffolds. The cells were pre-differentiated on scaffolds for one week and then bone formation *in vivo* was determined after subcutaneous implantation into immune-suppressed rats[89].

Again, human AFSCs were able to proliferate and differentiate into the osteogenic phenotype, producing mineralized extracellular matrix similar to BM-MSCs in 2D culture conditions[90]. Moreover, when seeded on microfibrous starch and a poly(ε-caprolactone) scaffold, AFSCs successfully differentiated into osteogenic cells. The high porosity of the scaffold influenced the sequential development of osteoblastic cells and improved the osteogenic differentiation process. Some differences from BM-MSCs were observed in the expression of RunX-2, collagen Ⅰ and ALP activity, denoting that cells from different origins may express different osteogenic markers at different times[90].

In addition, AFSCs were also able to produce mineralized matrix within porous medical-grade poly-e-caprolactone (mPCL) scaffold. The latter has good mechanical stability, large surface area and high porosity, allowing the attachment of AFSCs and extracellular matrix deposition. The construct, composed of an mPCL scaffold and AFSCs pre-differentiated for 28 d, was succeeded in producing seven times more mineralized matrix when implanted subcutaneously on the dorsal side of athymic female nude rats[91].

The same cells also proved able to adhere to a composite scaffold formed of a collagen matrix derived from porcine bladder submucosa matrix and poly(lactide-co-glycolide). In this condition, the osteogenic differentiation of AFSCs was improved, as revealed by increased mineralization and upregulation of osteogenic genes[92]. Indeed, the combination of natural scaffold with synthetic polymers was able to provide a microenvironment to facilitate the osteogenic differentiation.

More recently, a construct composed of collagen type I and AFSCs was used to evaluate bone regeneration in critical-sized rat calvarial defect after oral administration of phytoestrogen ferutinin[93]. Interestingly, 4 wk after implantation, the construct had reconstructed almost 70% of the critical-size defect[93]. Collagen type I, the major component of the extracellular matrix, facilitated cell adhesion and bone-forming cells in the defect site.

Another interesting study, by Mirabella *et al*[38] demonstrated that AFSCs loaded onto a biomaterial named Skelite and subcutaneously implanted in the back of CD-1 nu/nu mice gave rise to mineralized bone. Skelite disk is composed of silicon stabilized tricalcium phosphate biomaterial with a porosity of approximately 60% and an open structure similar to bone. However, they found that AFSCs did not have a direct role in new bone formation, but contributed to host progenitor recruitment and vessel formation in the engineered bone.

The unique features of AF-derived cells combined with the interesting results obtained in animal models of skeletal damage (Table 2) make them promising for the regeneration of bone tissue. Thanks to their immunosuppressive properties, allogeneic transplantation of AF-MSCs may be applied in various orthopedic conditions, and cell banks may be set up for regenerative medicine (Figure 1). However, more accurate understanding of the behavior and homing of AF-MSCs *in vivo* is necessary before they can be clinically applied. Moreover, additional studies are required to improve implanted cell survival and to ascertain the best biomaterial and the optimal combination of cytokines and growth factors.

## CONCLUSION

One purpose of bone regeneration is to find the most appropriate source of stem cells for clinical application together with the best bio-compatible support.

Bone marrow harvesting requires invasive procedures while the MSC number and differentiation potential decline with increasing age of the donor[94].

MSCs from extra-embryonic tissues are easily accessible. Moreover, their osteogenic potential and their safety in humans have been already tested. What is more, considering the high number of MSCs that can be isolated from amniotic fluid, a possibility of banking these cells adds greater promise of their clinical application. Furthermore, the osteogenic commitment of AF-MSCs could be enhanced by using appropriate osteoconductive scaffolds and osteoinductive growth factors[90].

Although these cells are very promising, therefore, in order to apply them for bone regeneration further investigations are needed to select the safest and most efficient cell-based approach. Certainly the results obtained so far are most promising: preclinical and clinical studies should be continued, thus opening new insights in the foreseeable future.

## REFERENCES


1 **Cauley JA**. Public health impact of osteoporosis. *J Gerontol A Biol Sci Med Sci* 2013; **68**: 1243-1251 [PMID: 23902935 DOI: 10.1093/gerona/glt093]

2 **Jimi E**, Hirata S, Osawa K, Terashita M, Kitamura C, Fukushima H. The current and future therapies of bone regeneration to repair bone defects. *Int J Dent* 2012; **2012**: 148261 [PMID: 22505894 DOI: 10.1155/2012/148261]

3 **Waese EY**, Kandel RA, Stanford WL. Application of stem cells in bone repair. *Skeletal Radiol* 2008; **37**: 601-608 [PMID: 18193216 DOI: 10.1007/s00256-007-0438-8]

4 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

5 **Liu ZJ**, Zhuge Y, Velazquez OC. Trafficking and differentiation of mesenchymal stem cells. *J Cell Biochem* 2009; **106**: 984-991 [PMID: 19229871 DOI: 10.1002/jcb.22091]

6 **Harada S**, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature* 2003; **423**: 349-355 [PMID: 12748654 DOI: 10.1038/nature01660]

7 **Bielby R**, Jones E, McGonagle D. The role of mesenchymal stem cells in maintenance and repair of bone. *Injury* 2007; **38** Suppl 1: S26-S32 [PMID: 17383482 DOI: 10.1016/j.injury.2007.02.007]

8 **Phimphilai M**, Zhao Z, Boules H, Roca H, Franceschi RT. BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J Bone Miner Res* 2006; **21**: 637-646 [PMID: 16598384 DOI: 10.1359/jbmr.060109]

9 **Rawadi G**, Vayssière B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Miner Res* 2003; **18**: 1842-1853 [PMID: 14584895 DOI: 10.1359/jbmr.2003.18.10.1842]

10 **Gimble JM**, Zvonic S, Floyd ZE, Kassem M, Nuttall ME. Playing with bone and fat. *J Cell Biochem* 2006; **98**: 251-266 [PMID: 16479589 DOI: 10.1002/jcb.20777]

11 **James AW**. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica* (Cairo) 2013; **2013**: 684736 [PMID: 24416618 DOI: 10.1155/2013/684736]

12 **Kang Q**, Song WX, Luo Q, Tang N, Luo J, Luo X, Chen J, Bi Y, He BC, Park JK, Jiang W, Tang Y, Huang J, Su Y, Zhu GH, He Y, Yin H, Hu Z, Wang Y, Chen L, Zuo GW, Pan X, Shen J, Vokes T, Reid RR, Haydon RC, Luu HH, He TC. A comprehensive analysis

of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. *Stem Cells Dev* 2009; **18**: 545-559 [PMID: 18616389 DOI: 10.1089/scd.2008.0130]

13 **D'Alimonte I**, Lannutti A, Pipino C, Di Tomo P, Pierdomenico L, Cianci E, Antonucci I, Marchisio M, Romano M, Stuppia L, Caciagli F, Pandolfi A, Ciccarelli R. Wnt signaling behaves as a "master regulator" in the osteogenic and adipogenic commitment of human amniotic fluid mesenchymal stem cells. *Stem Cell Rev* 2013; **9**: 642-654 [PMID: 23605563 DOI: 10.1007/s12015-013-9436-5]

14 **James AW**, Leucht P, Levi B, Carre AL, Xu Y, Helms JA, Longaker MT. Sonic Hedgehog influences the balance of osteogenesis and adipogenesis in mouse adipose-derived stromal cells. *Tissue Eng Part A* 2010; **16**: 2605-2616 [PMID: 20367246 DOI: 10.1089/ten.TEA.2010.0048]

15 **James AW**, Pang S, Askarinam A, Corselli M, Zara JN, Goyal R, Chang L, Pan A, Shen J, Yuan W, Stoker D, Zhang X, Adams JS, Ting K, Soo C. Additive effects of sonic hedgehog and Nell-1 signaling in osteogenic versus adipogenic differentiation of human adipose-derived stromal cells. *Stem Cells Dev* 2012; **21**: 2170-2178 [PMID: 22264144 DOI: 10.1089/scd.2011.0461]

16 **Knight MN**, Hankenson KD. Mesenchymal Stem Cells in Bone Regeneration. *Adv Wound Care* (New Rochelle) 2013; **2**: 306-316 [PMID: 24527352 DOI: 10.1089/wound.2012.0420]

17 **Caplan AI**. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng* 2005; **11**: 1198-1211 [PMID: 16144456]

18 **Wang X**, Wang Y, Gou W, Lu Q, Peng J, Lu S. Role of mesenchymal stem cells in bone regeneration and fracture repair: a review. *Int Orthop* 2013; **37**: 2491-2498 [PMID: 23948983 DOI: 10.1007/s00264-013-2059-2]

19 **Weissman IL**. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 2000; **287**: 1442-1446 [PMID: 10688785 DOI: 10.1126/science.287.5457.1442]

20 **da Silva Meirelles L**, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006; **119**: 2204-2213 [PMID: 16684817 DOI: 10.1242/jcs.02932]

21 **Zvaifler NJ**, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res* 2000; **2**: 477-488 [PMID: 11056678]

22 **Zhu X**, Shi W, Tai W, Liu F. The comparition of biological characteristics and multilineage differentiation of bone marrow and adipose derived Mesenchymal stem cells. *Cell Tissue Res* 2012; **350**: 277-287 [PMID: 22661317 DOI: 10.1007/s00441-012-1453-1]

23 **Park JR**, Kim E, Yang J, Lee H, Hong SH, Woo HM, Park SM, Na S, Yang SR. Isolation of human dermis derived mesenchymal stem cells using explants culture method: expansion and phenotypical characterization. *Cell Tissue Bank* 2014 Aug 28; Epub ahead of print [PMID: 25163610 DOI: 10.1007/s10561-014-9471-8]

24 **Wagner W**, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 2005; **33**: 1402-1416 [PMID: 16263424 DOI: 10.1016/j.exphem.2005.07.003]

25 **Campagnoli C**, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001; **98**: 2396-2402 [PMID: 11588036 DOI: 10.1182/blood.V98.8.2396]

26 **Guillot PV**, De Bari C, Dell'Accio F, Kurata H, Polak J, Fisk NM. Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation* 2008; **76**: 946-957 [PMID: 18557767 DOI: 10.1111/j.1432-0436.2008.00279.x]

27 **Fauza D**. Amniotic fluid and placental stem cells. *Best Pract Res Clin Obstet Gynaecol* 2004; **18**: 877-891 [PMID: 15582544 DOI: 10.1016/j.bpobgyn.2004.07.001]

28 **Pipino C**, Shangaris P, Resca E, Zia S, Deprest J, Sebire NJ, David AL, Guillot PV, De Coppi P. Placenta as a reservoir of stem cells: an underutilized resource? *Br Med Bull* 2013; **105**: 43-68 [PMID: 23184854 DOI: 10.1093/bmb/lds033]

29 **Secco M**, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M. Mesenchymal stem cells from umbilical cord: do not discard the cord! *Neuromuscul Disord* 2008; **18**: 17-18 [PMID: 18155523 DOI: 10.1016/j.nmd.2007.11.003]

30 **Abdulrazzak H**, Moschidou D, Jones G, Guillot PV. Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. *J R Soc Interface* 2010; **7** Suppl 6: S689-S706 [PMID: 20739312 DOI: 10.1098/rsif.2010.0347.focus]

31 **Bieback K**, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* 2004; **22**: 625-634 [PMID: 15277708 DOI: 10.1634/stemcells.22-4-625]

32 **Siegel N**, Rosner M, Hanneder M, Valli A, Hengstschläger M. Stem cells in amniotic fluid as new tools to study human genetic diseases. *Stem Cell Rev* 2007; **3**: 256-264 [PMID: 17955390 DOI: 10.1007/s12015-007-9003-z]

33 **Shaw SW**, Blundell MP, Pipino C, Shangaris P, Maghsoudlou P, Ramachandra DL, Georgiades F, Boyd M, Thrasher AJ, Porada CD, Almeida-Porada G, Cheng PJ, David AL, de Coppi P. Sheep CD34+ amniotic fluid cells have hematopoietic potential and engraft after autologous in utero transplantation. *Stem Cells* 2015; **33**: 122-132 [PMID: 25186828 DOI: 10.1002/stem.1839]

34 **Cananzi M**, Atala A, De Coppi P. Stem cells derived from amniotic fluid: new potentials in regenerative medicine. *Reprod Biomed Online* 2009; **18** Suppl 1: 17-27 [PMID: 19281660 DOI: 10.1016/S1472-6483(10)60111-3]

35 **Carraro G**, Perin L, Sedrakyan S, Giuliani S, Tiozzo C, Lee J, Turcatel G, De Langhe SP, Driscoll B, Bellusci S, Minoo P, Atala A, De Filippo RE, Warburton D. Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages. *Stem Cells* 2008; **26**: 2902-2911 [PMID: 18719226 DOI: 10.1634/stemcells.2008-0090]

36 **Bollini S**, Cheung KK, Riegler J, Dong X, Smart N, Ghionzoli M, Loukogeorgakis SP, Maghsoudlou P, Dubé KN, Riley PR, Lythgoe MF, De Coppi P. Amniotic fluid stem cells are cardioprotective following acute myocardial infarction. *Stem Cells Dev* 2011; **20**: 1985-1994 [PMID: 21534857 DOI: 10.1089/scd.2010.0424]

37 **De Coppi P**, Callegari A, Chiavegato A, Gasparotto L, Piccoli M, Taiani J, Pozzobon M, Boldrin L, Okabe M, Cozzi E, Atala A, Gamba P, Sartore S. Amniotic fluid and bone marrow derived mesenchymal stem cells can be converted to smooth muscle cells in the cryo-injured rat bladder and prevent compensatory hypertrophy of surviving smooth muscle cells. *J Urol* 2007; **177**: 369-376 [PMID: 17162093 DOI: 10.1016/j.juro.2006.09.103]

38 **Mirabella T**, Gentili C, Daga A, Cancedda R. Amniotic fluid stem cells in a bone microenvironment: driving host angiogenic response. *Stem Cell Res* 2013; **11**: 540-551 [PMID: 23584083 DOI: 10.1016/j.scr.2013.02.006]

39 **Bossolasco P**, Montemurro T, Cova L, Zangrossi S, Calzarossa C, Buiatiotis S, Soligo D, Bosari S, Silani V, Deliliers GL, Rebulla P, Lazzari L. Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. *Cell Res* 2006; **16**: 329-336 [PMID: 16617328 DOI: 10.1038/sj.cr.7310043]

40 **De Coppi P**, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S, Atala A. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007; **25**: 100-106 [PMID: 17206138 DOI: 10.1038/nbt1274]

41 **In 't Anker PS**, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, Fibbe WE, Kanhai HH. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 2003; **102**: 1548-1549 [PMID: 12900350 DOI: 10.1182/blood-2003-04-1291]

42 **Kaviani A**, Perry TE, Dzakovic A, Jennings RW, Ziegler MM, Fauza DO. The amniotic fluid as a source of cells for fetal tissue engineering. *J Pediatr Surg* 2001; **36**: 1662-1665 [PMID: 11685697 DOI: 10.1053/jpsu.2001.27945]

43 **Sessarego N**, Parodi A, Podestà M, Benvenuto F, Mogni M,

Raviolo V, Lituania M, Kunkl A, Ferlazzo G, Bricarelli FD, Uccelli A, Frassoni F. Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. *Haematologica* 2008; **93**: 339-346 [PMID: 18268281 DOI: 10.3324/haematol.11869]

44 **Pipino C**, Di Tomo P, Mandatori D, Cianci E, Lanuti P, Cutrona MB, Penolazzi L, Pierdomenico L, Lambertini E, Antonucci I, Sirolli V, Bonomini M, Romano M, Piva R, Marchisio M, Pandolfi A. Calcium sensing receptor activation by calcimimetic R-568 in human amniotic fluid mesenchymal stem cells: correlation with osteogenic differentiation. *Stem Cells Dev* 2014; **23**: 2959-2971 [PMID: 25036254 DOI: 10.1089/scd.2013.0627]

45 **Pozzobon M**, Ghionzoli M, De Coppi P. ES, iPS, MSC, and AFS cells. Stem cells exploitation for Pediatric Surgery: current research and perspective. *Pediatr Surg Int* 2010; **26**: 3-10 [PMID: 19727766 DOI: 10.1007/s00383-009-2478-8]

46 **Sarić T**, Frenzel LP, Hescheler J. Immunological barriers to embryonic stem cell-derived therapies. *Cells Tissues Organs* 2008; **188**: 78-90 [PMID: 18303241 DOI: 10.1159/000118784]

47 **Hentze H**, Graichen R, Colman A. Cell therapy and the safety of embryonic stem cell-derived grafts. *Trends Biotechnol* 2007; **25**: 24-32 [PMID: 17084475 DOI: 10.1016/j.tibtech.2006.10.010]

48 **Grinnemo KH**, Sylvén C, Hovatta O, Dellgren G, Corbascio M. Immunogenicity of human embryonic stem cells. *Cell Tissue Res* 2008; **331**: 67-78 [PMID: 17846795 DOI: 10.1007/s00441-007-0486-3]

49 **Daley GQ**, Ahrlund Richter L, Auerbach JM, Benvenisty N, Charo RA, Chen G, Deng HK, Goldstein LS, Hudson KL, Hyun I, Junn SC, Love J, Lee EH, McLaren A, Mummery CL, Nakatsuji N, Racowsky C, Rooke H, Rossant J, Schöler HR, Solbakk JH, Taylor P, Trounson AO, Weissman IL, Wilmut I, Yu J, Zoloth L. Ethics. The ISSCR guidelines for human embryonic stem cell research. *Science* 2007; **315**: 603-604 [PMID: 17272706]

50 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408]

51 **Okita K**, Yamanaka S. Induced pluripotent stem cells: opportunities and challenges. *Philos Trans R Soc Lond B Biol Sci* 2011; **366**: 2198-2207 [PMID: 21727125 DOI: 10.1098/rstb.2011.0016]

52 **Mukherjee S**, Thrasher AJ. iPSCs: Unstable origins? *Mol Ther* 2011; **19**: 1188-1190 [PMID: 21720379 DOI: 10.1038/mt.2011.116]

53 **Galende E**, Karakikes I, Edelmann L, Desnick RJ, Kerenyi T, Khoueiry G, Lafferty J, McGinn JT, Brodman M, Fuster V, Hajjar RJ, Polgar K. Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. *Cell Reprogram* 2010; **12**: 117-125 [PMID: 20677926 DOI: 10.1089/cell.2009.0077]

54 **Pipino C**, Mukherjee S, David AL, Blundell MP, Shaw SW, Sung P, Shangaris P, Waters JJ, Ellershaw D, Cavazzana M, Mostoslavsky G, Pandolfi A, Pierro A, Guillot PV, Thrasher AJ, De Coppi P. Trisomy 21 mid-trimester amniotic fluid induced pluripotent stem cells maintain genetic signatures during reprogramming: implications for disease modeling and cryobanking. *Cell Reprogram* 2014; **16**: 331-344 [PMID: 25162836 DOI: 10.1089/cell.2013.0091]

55 **Spinelli V**, Guillot PV, De Coppi P. Induced pluripotent stem (iPS) cells from human fetal stem cells (hFSCs). *Organogenesis* 2013; **9**: 101-110 [PMID: 23823661 DOI: 10.4161/org.25197]

56 **Moschidou D**, Mukherjee S, Blundell MP, Drews K, Jones GN, Abdulrazzak H, Nowakowska B, Phoolchund A, Lay K, Ramasamy TS, Cananzi M, Nettersheim D, Sullivan M, Frost J, Moore G, Vermeesch JR, Fisk NM, Thrasher AJ, Atala A, Adjaye J, Schorle H, De Coppi P, Guillot PV. Valproic acid confers functional pluripotency to human amniotic fluid stem cells in a transgene-free approach. *Mol Ther* 2012; **20**: 1953-1967 [PMID: 22760542 DOI: 10.1038/mt.2012.117]

57 **Ramachandra DL**, Shaw SS, Shangaris P, Loukogeorgakis S, Guillot PV, Coppi PD, David AL. In utero therapy for congenital disorders using amniotic fluid stem cells. *Front Pharmacol* 2014; **5**: 270 [PMID: 25566071 DOI: 10.3389/fphar.2014.00270]

58 **Mauro A**, Turriani M, Ioannoni A, Russo V, Martelli A, Di Giacinto O, Nardinocchi D, Berardinelli P. Isolation, characterization, and in vitro differentiation of ovine amniotic stem cells. *Vet Res Commun* 2010; **34** Suppl 1: S25-S28 [PMID: 20437094 DOI: 10.1007/s11259-010-9393-2]

59 **Liu ZS**, Xu YF, Feng SW, Li Y, Yao XL, Lu XL, Zhang C. Baculovirus-transduced mouse amniotic fluid-derived stem cells maintain differentiation potential. *Ann Hematol* 2009; **88**: 565-572 [PMID: 19066893 DOI: 10.1007/s00277-008-0634-1]

60 **Antonucci I**, Iezzi I, Morizio E, Mastrangelo F, Pantalone A, Mattioli-Belmonte M, Gigante A, Salini V, Calabrese G, Tetè S, Palka G, Stuppia L. Isolation of osteogenic progenitors from human amniotic fluid using a single step culture protocol. *BMC Biotechnol* 2009; **9**: 9 [PMID: 19220883 DOI: 10.1186/1472-6750-9-9]

61 **Liu Y**, Wu J, Zhu Y, Han J. Therapeutic application of mesenchymal stem cells in bone and joint diseases. *Clin Exp Med* 2014; **14**: 13-24 [PMID: 23124706 DOI: 10.1007/s10238-012-0218-1]

62 **Liao HT**, Chen CT. Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells. *World J Stem Cells* 2014; **6**: 288-295 [PMID: 25126378 DOI: 10.4252/wjsc.v6.i3.288]

63 **Barba M**, Pirozzi F, Saulnier N, Vitali T, Natale MT, Logroscino G, Robbins PD, Gambotto A, Neri G, Michetti F, Pola E, Lattanzi W. Lim mineralization protein 3 induces the osteogenic differentiation of human amniotic fluid stromal cells through Kruppel-like factor-4 downregulation and further bone-specific gene expression. *J Biomed Biotechnol* 2012; **2012**: 813894 [PMID: 23097599 DOI: 10.1155/2012/813894]

64 **de Lara Janz F**, Favero GM, Bohatch MS, Aguiar Debes A, Bydlowski SP. Simvastatin induces osteogenic differentiation in human amniotic fluid mesenchymal stem cells (AFMSC). *Fundam Clin Pharmacol* 2014; **28**: 211-216 [PMID: 23094676 DOI: 10.1111/fcp.12006]

65 **Liu M**, Li Y, Yang ST. Curculigoside improves osteogenesis of human amniotic fluid-derived stem cells. *Stem Cells Dev* 2014; **23**: 146-154 [PMID: 24007307 DOI: 10.1089/scd.2013.0261]

66 **Liu M**, Li Y, Yang ST. Effects of naringin on the proliferation and osteogenic differentiation of human amniotic fluid-derived stem cells. *J Tissue Eng Regen Med* 2014 Jun 11; Epub ahead of print [PMID: 24915843 DOI: 10.1002/term.1911]

67 **Zavatti M**, Resca E, Bertoni L, Maraldi T, Guida M, Carnevale G, Ferrari A, De Pol A. Ferutinin promotes proliferation and osteoblastic differentiation in human amniotic fluid and dental pulp stem cells. *Life Sci* 2013; **92**: 993-1003 [PMID: 23583571 DOI: 10.1016/j.lfs.2013.03.018]

68 **Di Tomo P**, Pipino C, Lanuti P, Morabito C, Pierdomenico L, Sirolli V, Bonomini M, Miscia S, Mariggiò MA, Marchisio M, Barboni B, Pandolfi A. Calcium sensing receptor expression in ovine amniotic fluid mesenchymal stem cells and the potential role of R-568 during osteogenic differentiation. *PLoS One* 2013; **8**: e73816 [PMID: 24040082 DOI: 10.1371/journal.pone.0073816]

69 **De Rosa A**, Tirino V, Paino F, Tartaglione A, Mitsiadis T, Feki A, d'Aquino R, Laino L, Colacurci N, Papaccio G. Amniotic fluid-derived mesenchymal stem cells lead to bone differentiation when cocultured with dental pulp stem cells. *Tissue Eng Part A* 2011; **17**: 645-653 [PMID: 20919950 DOI: 10.1089/ten.TEA.2010.0340]

70 **Choi E**, Choi E, Hwang KC. MicroRNAs as novel regulators of stem cell fate. *World J Stem Cells* 2013; **5**: 172-187 [PMID: 24179605 DOI: 10.4252/wjsc.v5.i4.172]

71 **Trohatou O**, Zagoura D, Bitsika V, Pappa KI, Antsaklis A, Anagnou NP, Roubelakis MG. Sox2 suppression by miR-21 governs human mesenchymal stem cell properties. *Stem Cells Transl Med* 2014; **3**: 54-68 [PMID: 24307698 DOI: 10.5966/sctm.2013-0081]

72 **Chen Q**, Xiao P, Chen JN, Cai JY, Cai XF, Ding H, Pan YL. AFM studies of cellular mechanics during osteogenic differentiation of human amniotic fluid-derived stem cells. *Anal Sci* 2010; **26**: 1033-1037 [PMID: 20953044]

73 **Macsai CE**, Foster BK, Xian CJ. Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair. *J Cell Physiol* 2008; **215**: 578-587 [PMID: 18247365 DOI: 10.1002/jcp.21342]

74 **Liu G**, Vijayakumar S, Grumolato L, Arroyave R, Qiao H, Akiri G, Aaronson SA. Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells. *J Cell Biol* 2009; **185**: 67-75 [PMID: 19349579 DOI: 10.1083/jcb.200810137]

75 **Alfadda TI**, Saleh AM, Houillier P, Geibel JP. Calcium-sensing receptor 20 years later. *Am J Physiol Cell Physiol* 2014; **307**: C221-C231 [PMID: 24871857 DOI: 10.1152/ajpcell.00139.2014]

76 **Yamaguchi T**. The calcium-sensing receptor in bone. *J Bone Miner Metab* 2008; **26**: 301-311 [PMID: 18600395 DOI: 10.1007/s00774-008-0843-7]

77 **Marie PJ**. The calcium-sensing receptor in bone cells: a potential therapeutic target in osteoporosis. *Bone* 2010; **46**: 571-576 [PMID: 19660583 DOI: 10.1016/j.bone.2009.07.082]

78 **Oryan A**, Alidadi S, Moshiri A, Maffulli N. Bone regenerative medicine: classic options, novel strategies, and future directions. *J Orthop Surg Res* 2014; **9**: 18 [PMID: 24628910 DOI: 10.1186/1749-799X-9-18]

79 **Ma PX**. Biomimetic materials for tissue engineering. *Adv Drug Deliv Rev* 2008; **60**: 184-198 [PMID: 18045729 DOI: 10.1016/j.addr.2007.08.041]

80 **Osugi M**, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. *Tissue Eng Part A* 2012; **18**: 1479-1489 [PMID: 22443121 DOI: 10.1089/ten.TEA.2011.0325]

81 **Hilfiker A**, Kasper C, Hass R, Haverich A. Mesenchymal stem cells and progenitor cells in connective tissue engineering and regenerative medicine: is there a future for transplantation? *Langenbecks Arch Surg* 2011; **396**: 489-497 [PMID: 21373941 DOI: 10.1007/s00423-011-0762-2]

82 **Meirelles Lda S**, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* 2009; **20**: 419-427 [PMID: 19926330 DOI: 10.1016/j.cytogfr.2009.10.002]

83 **Kikuchi M**. Hydroxyapatite/collagen bone-like nanocomposite. *Biol Pharm Bull* 2013; **36**: 1666-1669 [PMID: 24189409]

84 **Pastorino L**, Dellacasa E, Scaglione S, Giulianelli M, Sbrana F, Vassalli M, Ruggiero C. Oriented collagen nanocoatings for tissue engineering. *Colloids Surf B Biointerfaces* 2014; **114**: 372-378 [PMID: 24246194 DOI: 10.1016/j.colsurfb.2013.10.026]

85 **Berardinelli P**, Valbonetti L, Muttini A, Martelli A, Peli R, Zizzari V, Nardinocchi D, Vulpiani MP, Tetè S, Barboni B, Piattelli A, Mattioli M. Role of amniotic fluid mesenchymal cells engineered on MgHA/collagen-based scaffold allotransplanted on an experimental animal study of sinus augmentation. *Clin Oral Investig* 2013; **17**: 1661-1675 [PMID: 23064983 DOI: 10.1007/s00784-012-0857-3]

86 **Sun H**, Feng K, Hu J, Soker S, Atala A, Ma PX. Osteogenic differentiation of human amniotic fluid-derived stem cells induced by bone morphogenetic protein-7 and enhanced by nanofibrous scaffolds. *Biomaterials* 2010; **31**: 1133-1139 [PMID: 19857889 DOI: 10.1016/j.biomaterials.2009.10.030]

87 **Woo KM**, Jun JH, Chen VJ, Seo J, Baek JH, Ryoo HM, Kim GS, Somerman MJ, Ma PX. Nano-fibrous scaffolding promotes osteoblast differentiation and biomineralization. *Biomaterials* 2007; **28**: 335-343 [PMID: 16854461 DOI: 10.1016/j.biomaterials.2006.06.013]

88 **Kolambkar YM**, Peister A, Ekaputra AK, Hutmacher DW, Guldberg RE. Colonization and osteogenic differentiation of different stem cell sources on electrospun nanofiber meshes. *Tissue Eng Part A* 2010; **16**: 3219-3230 [PMID: 20504075 DOI: 10.1089/ten.TEA.2010.0004]

89 **Maraldi T**, Riccio M, Resca E, Pisciotta A, La Sala GB, Ferrari A, Bruzzesi G, Motta A, Migliaresi C, Marzona L, De Pol A. Human amniotic fluid stem cells seeded in fibroin scaffold produce in vivo mineralized matrix. *Tissue Eng Part A* 2011; **17**: 2833-2843 [PMID: 21864161 DOI: 10.1089/ten.tea.2011.0062]

90 **Rodrigues MT**, Lee SJ, Gomes ME, Reis RL, Atala A, Yoo JJ. Amniotic fluid-derived stem cells as a cell source for bone tissue engineering. *Tissue Eng Part A* 2012; **18**: 2518-2527 [PMID: 22891759 DOI: 10.1089/ten.tea.2011.0672]

91 **Peister A**, Deutsch ER, Kolambkar Y, Hutmacher DW, Guldberg RE. Amniotic fluid stem cells produce robust mineral deposits on biodegradable scaffolds. *Tissue Eng Part A* 2009; **15**: 3129-3138 [PMID: 19344289 DOI: 10.1089/ten.TEA.2008.0536]

92 **Kim J**, Jeong SY, Ju YM, Yoo JJ, Smith TL, Khang G, Lee SJ, Atala A. In vitro osteogenic differentiation of human amniotic fluid-derived stem cells on a poly(lactide-co-glycolide) (PLGA)-bladder submucosa matrix (BSM) composite scaffold for bone tissue engineering. *Biomed Mater* 2013; **8**: 014107 [PMID: 23353783 DOI: 10.1088/1748-6041/8/1/014107]

93 **Zavatti M**, Bertoni L, Maraldi T, Resca E, Beretti F, Guida M, La Sala GB, De Pol A. Critical-size bone defect repair using amniotic fluid stem cell/collagen constructs: effect of oral ferutinin treatment in rats. *Life Sci* 2015; **121**: 174-183 [PMID: 25445219 DOI: 10.1016/j.lfs.2014.10.020]

94 **Rosset P**, Deschaseaux F, Layrolle P. Cell therapy for bone repair. *Orthop Traumatol Surg Res* 2014; **100**: S107-S112 [PMID: 24411717 DOI: 10.1016/j.otsr.2013.11.010]


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REVIEW

# Stem cell therapy in the management of shoulder rotator cuff disorders

Maria Valencia Mora, Miguel A Ruiz Ibán, Jorge Díaz Heredia, Raul Barco Laakso, Ricardo Cuéllar, Mariano García Arranz

Maria Valencia Mora, Miguel A Ruiz Ibán, Jorge Díaz Heredia, Unidad de Hombro y Codo, Hospital Universitario Ramón y Cajal, 28034 Madrid, Spain
Raul Barco Laakso, Hospital Universitario La Paz, 28046 Madrid, Spain
Ricardo Cuéllar, Departamento de Traumatología y Cirugía Ortopédica, Hospital Universitario Donostia, 20080 San Sebastián, Spain
Mariano García Arranz, Instituto de Investigación Sanitaria Fundación Jiménez Diaz, 28040 Madrid, Spain
Mariano García Arranz, Departamento de Cirugía, Universidad Autónoma de Madrid, 28049 Madrid, Spain
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Conflict-of-interest: Mariano García Arranz has the following conflict of interest: MGA is inventor on 2 patents entitled "Identification and isolation of multipotent cells from non-osteochondral mesenchymal tissue" (10157355957US) and "Use of adipose tissue-derived stromal stem cells in treating fistula" (US11/167061). The Universidad Autónoma de Madrid (UAM) and Cellerix share patent rights. The rest of the authors do not have any conflict of interest.
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Correspondence to: Miguel A Ruiz Ibán, MD, PhD, Unidad de Hombro y Codo, Hospital Universitario Ramón y Cajal, Ctra. de Colmenar Viejo km. 9, 100, 28034 Madrid, Spain. drmri@hotmail.com
Telephone: +34-91-3368000
Fax: +34-91-3368000
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## Abstract

Rotator cuff tears are frequent shoulder problems that are usually dealt with surgical repair. Despite improved surgical techniques, the tendon-to-bone healing rate is unsatisfactory due to difficulties in restoring the delicate transitional tissue between bone and tendon. It is essential to understand the molecular mechanisms that determine this failure. The study of the molecular environment during embryogenesis and during normal healing after injury is key in devising strategies to get a successful repair. Mesenchymal stem cells (MSC) can differentiate into different mesodermal tissues and have a strong paracrine, anti-inflammatory, immunoregulatory and angiogenic potential. Stem cell therapy is thus a potentially effective therapy to enhance rotator cuff healing. Promising results have been reported with the use of autologous MSC of different origins in animal studies: they have shown to have better healing properties, increasing the amount of fibrocartilage formation and improving the orientation of fibrocartilage fibers with less immunologic response and reduced lymphocyte infiltration. All these changes lead to an increase in biomechanical strength. However, animal research is still inconclusive and more experimental studies are needed before human application. Future directions include expanded stem cell therapy in combination with growth factors or different scaffolds as well as new stem cell types and gene therapy.

**Key words:** Rotator cuff; Enthesis; Biologic; Stem cells

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**Core tip:** Current surgical techniques in rotator cuff repair do not achieve good tendon-to-bone healing. The use of stem cells to improve healing is a promising alternative. Different *in vivo* animal studies have shown

good results in achieving restoration of the native enthesis. However, human studies are scarce so the use of stem cell therapy in rotator cuff repair should still be considered and experimental technique. Further basic and clinical research is needed.

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## INTRODUCTION

The rotator cuff is a structure formed by the tendinous insertions of a group of muscles that dynamically stabilize the glenohumeral joint. Rotator cuff disease is the most common condition of the shoulder for which patients seek treatment and can be found in 30% to 50% of the population aged older than 50 years[1,2]. However, it also affects athletes and active individuals regardless of age and activity level.

Rotator cuff tears often require surgical treatment in order to increase function and decrease pain[3,4]. The objective of the treatment is the repair of the damaged tendons. Whether or not healing of the tear is a prognostic factor on function and pain after rotator cuff repair has been controversial. However, most of the authors have found that tear recurrence determines lower functional scores and a decrease in patient satisfaction[5-7]. In an attempt to improve the strength of the surgical repair, new materials and surgical techniques that aim to reproduce the anatomical footprint of the rotator cuff have been proposed[8,9]. Despite these significant technical advances, several studies have shown a persistently high failure rate of tendon to bone rotator cuff repair that ranges from 30% to 94%[6,10,11].

The main problem with failure in rotator cuff repair is probably biologic, as it is well known that the delicate and highly specialized fibro-cartilaginous transition zone between the rotator cuff and the bone does not regenerate after repair[12,13]. Standard tendon to bone repair techniques attain only a fibro-vascular scar tissue that has relatively poor mechanical properties[14]. Thus, the focus in research has changed from mechanical improvement of the repair techniques to finding ways to improve the biological environment around that repair[15-22]. This would include growth factors (GF), bone morphogenetic proteins (BMPs) as well as stem cells. The hypothesis is that biological therapies might facilitate the regeneration of the normal tendon-to-bone insertion microarchitecture and limit the amount of scar tissue. In this direction, isolated GF or platelet rich plasma has been recently used with variable results but stems cell are a more promising alternative[23-25].

**Table 1 Main biochemical and histological characteristics of the four areas of the enthesis[28]**

| Zone | Histological characteristics | Collagen type | Extracellular matrix composition |
|---|---|---|---|
| Zone 1 | Tendon | I | Decorin |
| Zone 2 | Non-mineralized fibrocartilage | II and III ( small amounts of I, IX and X) | Aggrecan and decorin |
| Zone 3 | Mineralized fibrocartilage | II (small amounts of X) | Aggrecan and mineral component |
| Zone 4 | Bone | I | Mineral component |

Stem cells have demonstrated great potential in enhancing the biologic healing process based on their influence in angiogenesis and the inflammatory pattern[26]. However, several questions still remain before they can be used clinically for augmenting tendon to bone healing. The purpose of this paper is to outline the current knowledge on the role stem cell therapy might have in dealing with rotator cuff tears and the future implications of the ongoing research's results.

## ENTHESIS: TENDON TO BONE HEALING IN THE ROTATOR CUFF

Tissue regeneration in the tendon-to-bone interphase is a complex process. The stiffness difference between tendon and bone is responsible for significant mechanical stress in the regeneration zone[27]. The enthesis represents a transitional tissue that allows for efficient energy transmission due to the gradual changes that occur in its microstructure, its histological characteristics and its biomechanical behaviour.

The enthesis has been divided into four zones: tendon, non-mineralized fibrocartilage, mineralized fibrocartilage and bone[28] (Figure 1 and Table 1). In the tendon area (zone 1) there is a predominance of type Ⅰ collagen fibres together with a small amount of decorin which is a small cellular or pericellular matrix proteoglycan; In the non-mineralized fibrocartilage area (zone 2), type Ⅱ and Ⅲ collagen fibres are predominant and small amounts of type Ⅰ, Ⅸ and Ⅹ collagen fibres have also been detected. Aggregans and decorine are also present. Zone 3 is constituted by the mineralized fibrocartilage, with a highly specialized mineralized content and type Ⅰ collagen fibres. Lastly, zone 4 is characterized for a bone-alike composition, as it corresponds to the bony insertional area. As previously mentioned, it has been demonstrated that this specialized tissue does not regenerate after injury and repair. The fibro vascular tissue that substitutes the native enthesis is characterized by a predominance of type III collagen due to the excessive formation of scar tissue and the absence of fibrocartilage.

The reparative process can be divided into 3 phases (inflammatory, reparative and remodelling) and numerous cells and cytokines have been

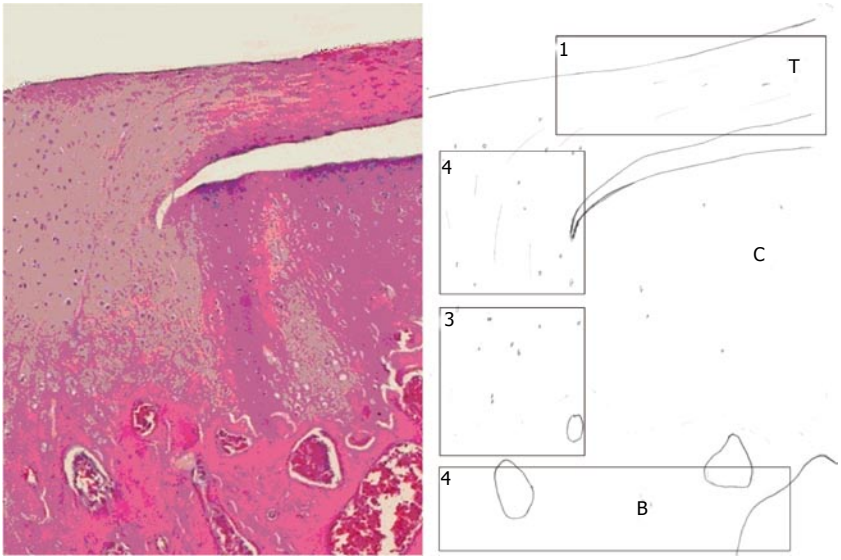


**Figure 1 The normal enthesis (longitudinal image and diagram of the bone-tendon junction of the supraspinatus tendon of a rat; hematoxilin-eosine, × 10): the supraspinatus tendon (T) approaches the humeral bone (B) immediately adyacent to the normal carlilage (C). The normal tendon (zone 1) gradually transforms into a fibrocartilaginous tissue with large mononucleated cells (zone 2).** As the fibers progress into the bone the extracellular matrix is progresivelyy calcified (zone 3) until it turns into normal bone (zone 4). Further explanation of the biochemical environment of these zones is shown in Table 1.

implicated[13,29,30]. Diaz-Heredia *et al*[30] have studied the gradual variation of vascular endothelial growth factor (VEGF), interleukin-1 (IL-1) and transforming growth factor-β1 (TGF-β1) in an animal model of rotator cuff tears in rats. Some authors have pointed out that the inability to regenerate the native enthesis could be caused by the incomplete expression of the genes implicated in its formation[28]. During embryogenic development, healing occurs without expression of TGF-β1 but with expression of TGF-β3, which determines an absence of scar tissue. On the contrary, during postnatal life, TGF-β1 is active during the three phases of the healing process[13,30,31]. Another important group of factors widely studied are BMP-12, 13 and 14 as well as fibroblast growth factor-β (FGF-β and insulin like growth factor-1 (IGF-1). Matrix metalloproteinases are multi-domain proteinases regulated by tissue inhibitors of metalloproteinases (TIMPs) and play a determinant role in the remodelling phase.

The enthesis structure is developed successfully during embryogenic period so knowledge of the biological mechanism of its development could help in pinpointing which factors are relevant in trying to regenerate the native transitional tissue[28]. Galatz *et al*[32] found that the mature fibrocartilage does not appear until 21 d after birth. Supraspinatus fibroblasts expressed type Ⅰ collagen during all the process. Type Ⅱ collagen was expressed firstly in the non-mineralized fibrocartilage and at 7 d in the mineralized fibrocartilage, where it persisted until 56 d. Type X collagen was initially seen in mineralized collagen at 14 d and it persisted until 56 d. There was a change in the presence from TGF-β3 to TGF-β1 at 15 d. The gradual expression of different factors present in the development of the physeal plate as (sex determining region Y)-box 9, Scleraxis, Patched 1, Parathyroid hormone-related protein (PTHrP) and Indian Heddegog (Ihh) has also been studied[32-34]. It has been proposed that the stratification in the structure and composition along the different zones of the enthesis could be a consequence of the gradual expression of these and other factors. For example, the amount of mineral deposit in the mature enthesis could be determined by the presence of osteogenic factors such as runt-related transcription factor 2 (Runx2) and bone morphogenetic protein-2 (BMP2). On the other hand, the formation of fibrocartilage could be related to a greater expression of PTHrP, Ihh and Sox9. Lastly, tendon development would be conditioned by the expression of BMP-12, tenomoduline and scleraxis[32]. Scleraxis is a protein member of the basic helix-loop-helix superfamily of transcription factors.

## BIOLOGIC APPROACH

In the past decades, as mentioned before, numerous biology-based strategies have been developed in order to improve the rate and quality of healing in rotator cuff models. The main areas of research, apart from stem cells, are matrix metalloproteinase (MMP) inhibitors and GF.

### *MMP inhibitors*

MMP expression is increased in degenerative rotator cuff tissue and it is known to cause progressive weakness in extracellular matrix. They are involved in tumoral growth, aneurysmatic disease and post-surgical tissue remodelling in the rotator cuff[35-37]. Tissular metalloproteinase inhibitors are thus, potential biological tools. In particular, inhibition of MMP-13, a MMP that is increased in degenerative rotator cuff tears, allows for higher amount of fibrocartilage formation, better collagen fiber organization and higher load to ultimate failure in the enthesis[36,38].

### *GF*

GF factors are key in the development of the different enthesis zones. The regeneration of the most specialized zone, the mineralized fibrocartilage, can be stimulated by osteoinductive factors[39]. The GF are

usually delivered with a vehicle, such as augmented sutures, fibrin gels or collagen sponges[16,40,41]. Rodeo *et al*[16] developed an animal model of supraspinatus repair in sheep in which they used BMP-2 to 7, TGFβ1, TGFβ2, TGF-β3 and FGF. They detected better histologic and biomechanical properties[16]. Other investigators have obtained similar results with BMP-12[42], BMP-13[17], BMP-14[43], FGF[40,44], IGF-1[45] and PDGF-b[15]. Some of these factors seem to play different roles depending in which zone of the enthesis they act or the timing of their effects.

The most widespread treatment, however, is platelet rich plasma (PRP) obtained from autologous blood[46]. It has been proposed that PRP facilitates coagulation and homeostasis, stimulates wound closure, restores intraarticular hyaluronic acid, equilibrates angiogenesis, promotes glucosamine synthesis and serves as a cellular support for migration and differentiation[23]. Despite the variable results obtained, it has been used for muscular, ligamentous, tendinous or cartilaginous injuries[47-50]. With regards to its application in rotator cuff tears, the results have also been controversial. Neither Sánchez Márquez *et al*[24] or Ruiz-Moneo *et al*[51] found any relevant clinical improvement with the use of PRP to augment suture in massive tears. However, other investigators support the use of PRP in selected cases[52]. For example, Randelli *et al*[25] in a prospective randomized clinical trial, found less postoperative pain and accelerated healing rate in patients with non-massive rotator cuff tears but there were no differences in functional scores and re-rupture rate. Due to the chronic nature of these injuries, it has been suggested that PRP application should be serial in order to enhance its benefits[53]. Another explanation for this fact is that the expression of growth factors is ephemeral. In this context, stem cell and gene therapies could be a more definitive and long-lasting treatment.

## STEM CELL THERAPY: ANIMAL STUDIES

The use of stem cell therapy in the regeneration of musculoskeletal tissue is a very dynamic field. MSCs of different origins, with their innate ability to differentiate into several mesenchymal tissues including bone, fat, muscle and tendon have been used extensively in tissue repair. Applications in which its usefulness has been confirmed are: treatment of bone defects, cartilage regeneration, meniscal regeneration and healing, management of tendinopathies and management of muscle lesions[54-56]. Investigators usually prefer adult MSCs over embryonic of fetal stem cells as the former are usually locally available and easier to obtain for the treatment of these non-life-threatening problems. Furthermore, the low immunogenicity of MSCs allows for the use of allogenic strains[57].

Some authors have also performed extensive research in animal bone-to-tendon healing models. Until recently the most widespread model reproduced the integration of anterior cruciate ligament tendinous grafts in a bone tunnel. In this animal model, the hamstring grafts are introduced into bony tunnels in both femoral and tibiae bones and pull out strength is tested. Lim *et al*[58] have used MSCs in this model in rabbits and found a significant increase in maximum load to failure.

It was not until 2009 that MSC therapy was applied to a rotator cuff model, since then the available literature has grown consistently. MSCs of different origins have been used for rotator cuff repair. Different tissue sources have been identified: bone marrow, adipose tissue, muscle, synovia, periosteum, tendon, dermis and umbilical cord or peripheral blood, have all been evaluated as sources of multipotent and pluripotent cell[26]. Although generally speaking MSCs of different origins have similar biological potential, there is increasing knowledge that certain MSC populations are better than others for specific tissue regeneration[59-61]. Table 2 shows the main animal investigations performed on rotator cuff repair.

### *Bone marrow MSCs*

The principal source for stem cell-enhanced healing of the rotator cuff has been autologous bone marrow (BM-MSCs). Gulotta *et al*[20] performed an experimental unilateral detachment of supraspinatus tendon and a transosseous repair in rats. BM-MSCs were harvested by performing lavage of intramedullary canals of long bones with Hank's Balanced Salt Solution (Gibco, Gaithersburg, MD). They showed that MSCs were present at the repair site and that they were metabolically active. Although they did not find significant differences in between the treated and untreated groups, at 4 wk, there was a higher amount of fibrocartilage formation and better orientation of fibrocartilage fibers.

In order to reproduce rotator cuff surgery, Kida *et al*[62] designed a study in which they performed additional drilling to the greater tuberosity to release bone marrow and allow bone marrow cells to migrate into the suture zone. They tested chimeric rats that expressed green fluorescent protein in the bone marrow cells and looked for the expression of this protein after a period of 2, 4 and 8 wk. It seems that drilling and the subsequent migration of stem cells might improve maximum load to failure at 4 and 8 wk.

More recently, Gulotta *et al*[63-65] have used genetically modified MSCs in order to express scleraxis and produce MIT1 and BMP-13 with promising results. MSCs genetically modified to over-express MT1-MMP might be useful for augmenting suture as it has demonstrated improved biomechanical strength at 4 wk based on a higher presence of fibrocartilage[63]. Results of studies with application of MSCs genetically modified to overexpress BMP-13[64] were not that successful. On the contrary, MSCs genetically modified with Scx demonstrated to promote better biomechanical

**Table 2 Rotator cuff repair animal models using mesenchimal stem cells**

| Ref. | Animal | Type of cells | Tendon repair model | Method of delivery | Results |
|---|---|---|---|---|---|
| Gulotta *et al*[20] | Rat | Allogenic BM-MSC | Supraspinatus tendon Acute repair | Fibrin glue carrier | No differences in structure, composition or strength at the repair site |
| Gulotta *et al*[63] | Rat | Allogenic BM-MSCs transduced with MT1-MMP | Supraspinatus tendon Acute repair | Fibrin glue carrier | Improved fibrocartilage Improved biomechanical strength |
| Gulotta *et al*[64] | Rat | Allogenic BM-MSCs transduced with human BMP-13 | Supraspinatus tendon Acute repair | Fibrin glue carrier | No differences in structure, composition or strength at the repair site |
| Gulotta *et al*[65] | Rat | Allogenic BM-MSCs transduced with sleraxis | Supraspinatus tendon Acute repair | Fibrin glue carrier | Improved fibrocartilage Improved mechanical resistance and stiffness |
| Shen *et al*[74] | Rabbit | Allogenic T-MSCs | Supraspinatus tendon Acute repair | Seeded scaffold (silk-collagen) | T-MSCs differentiated into tenocytes Improved collagen content Improved biological environment Less inflammation |
| Kida *et al*[62] | Rat | Autologous BM-MSC | Supraspinatus tendon Acute repair | Transosseous drilling | BM-MSCs infiltrated the repaired tendon Improved mechanical resistance |
| Oh *et al*[71] | Rabbit | Allogenic A-MSCs | Subscapularis tendon Chronic repair | Injection | Improved muscle function Improved tendon healing Decreased fatty infiltration |

Different types of cells have been used: Bone marrow derived MSCs (BM-MSCs), Tendon derived MSCs (T-MSCs) or Adipose derived MSCs (A-MSCs). MT1-MMP: Metalloproteinase inhibitor-1; BMP-13: Bone morphogenetic protein-13; MSCs: Mesenchymal stem cells.

characteristics at 2 wk[65].

### *Adipose tissue MSC*

Adipose Tissue derived stem cells (AMSC) have also shown multipotentiality *in vitro*[66]. Due to its mesodermal origin, they can differentiate into adipose lineage cells[67], osteogenic cells[68], chondrogenic cells[59] and myogenic cells[69]. *In vivo*, they have also demonstrated their capacity to differentiate into adipose tissue using different scaffolds as polyglicolic acid, collagen sponges or fibrin gel[54,70,71].

Recently, Oh *et al*[71] have published the first study in a rotator cuff model using AMSCs. Four groups were compared for a suture of the subscapularis tendon in rabbit using saline, saline and AMSCs, only AMSCs and only suture. They found better healing properties and a capacity of regeneration after fatty infiltration of the muscle.

### *MSCs of other origins (non-hematopoyetic)*

Muscle-derived stem cells (M-MSCs) have been isolated using a modification of a method known as the preplate technique[72]. Pelinkovic *et al*[73] have shown that the injection of M-MSCs into the supraspinatus tendon of athymic rats resulted in the engraftment of transplanted cells in a pattern with a morphology comparable to resident tendon fibers. The authors suggest that more studies are necessary before assuming that M-MSCs can improve rotator cuff healing.

Lastly, Shen *et al*[74] performed a study using tenocyte-derived stem cells (T-MSCs) proliferated *in vitro* and obtained from human fetal Achilles tendon samples. Implantation of this type of cells in the rabbit rotator cuff defect did not elicit an immunologic response but increased fibroblastic cell ingrowth and reduced infiltration of lymphocytes.

### *Choice of scaffold for MSCs deployment*

Cell adhesion to the scaffold depends on the interaction that is established in between the scaffold microstructure and the cell surface receptors denominated integrins. Transmembrane contacts are key factor for MSC survival, proliferation and differentiation[75]. Numerous studies have investigated the behaviour of stem cells in different scaffolds and have demonstrated that the scaffold can determine the differentiation capacity into one or other lineages[75]. Two different types of interactions have been described: physical and biochemical. Vehicles that maintain the rounded shape of the cells and avoid contact in between them, promote the chondrogenic differentiation and avoid expression of type I collagen. Porous gelatine vehicles or those that use fibrin favour a fibro cartilaginous phenotype due to the expression of collagen types I and II[76].

## STEM CELL THERAPY: HUMAN STUDIES

Although there is a lack of consensus on whether the application of stem cells to enhance the rotator cuff healing is effective or not, some authors have started developing different strategies for the clinical application of the experimental findings.

Beitzel *et al*[77] studied the quantity and characteristics of BM-MSCs obtained from proximal humerus and distal femur bone marrow aspiration and found them comparable, supporting the previous experimental research by Kida *et al*[62]. Rotator cuff derived MSCs have been isolated and compared to BM-derived stem

cells. It seems that the myogenic potential of MSCs derived from rotator cuff cells is higher than for BM-MSCs[78]. Randelli *et al*[79] could isolate tenocyte-derived stem cells from supraspinatus tendon and long head of biceps tendon. Utsunomiya *et al*[80] also studied the subacromial bursa as a potential source for MSCs and found that the synovial cells found in the bursa were a good cell source.

Ellera Gomes *et al*[81] published their work in 14 patients with a complete tear of the rotator cuff that was repaired in a trans osseous fashion through a mini-incision augmenting the suture with mononuclear stem cells from iliac crest bone marrow aspirate. At 12 mo, 12 of the 14 tears had healed according to clinical and magnetic resonance imaging results[81]. This is the only published investigation on clinical application of stem cells in rotator cuff tears.

Lastly, Beitzel *et al*[75] have also focused their attention in how different scaffolds behave in humans in order to extrapolate results obtained from experimental research. MSCs adhesion, proliferation, and scaffold morphology were evaluated by histologic analysis and electron microscopy. According to their findings, significant differences existed: non cross-linked porcine collagen scaffolds showed superior results for cell adhesion and proliferation, as well as on histologic evaluation.

## FUTURE ALTERNATIVES

Advanced stem cell therapy and gene therapy represent the most feasible option in order to improve rotator cuff healing[21]. A better knowledge of the molecular phases of embryogenesis of the enthesis as well as the injury and healing patterns have allowed to identify the growth factors and proteins to target[13,28].

A combination of stem cells, modified before implantation, using exposure to different growth factors or modifications to the culture conditions to generate a desired phenotype is one of the most investigated pathways[26]. Moreover, the newly recognized anti-inflammatory and antiapoptotic impact of MSCs on tissue healing may provide a great potential for functional restoration[76,82].

On the other hand, specific growth factor supplementation, in the form of transgenic therapy may allow longer-term tendon repair and potential return to function. Fetal-derived embryonic stem cell-like cells have recently been evaluated for tendon and ligament repair. More recently, induced pluripotent stem cells, developed by genetically reprogramming adult-sourced cells, may be particularly beneficial in the challenging environment of rotator cuff injury. Generation of iPS cells can use viral or, more recently, nonviral vector delivery of reprogramming genes. However, these transgenic therapies lack safety clearance when it comes to oncologic and teratogenic risks[26].

Lastly, stem cells associated to bio or nanotechnology can control the proliferation and differentiation into complex, viable 3D tissues. So we might be able to use biodegradable polymer scaffolds to promote cell growth and differentiation and formation of 3D structures. This could be useful in order to avoid scarring during the healing process.

## CONCLUSION

Current literature regarding the clinical use of stem cells in rotator cuff tears is limited. Although in vivo animal studies have shown promising results to enhance tendon-to-bone healing, the use of stem cell therapy in rotator cuff should still be considered an experimental technique. Further basic and clinical research is needed.

## REFERENCES


1 **Gomoll AH**, Katz JN, Warner JJ, Millett PJ. Rotator cuff disorders: recognition and management among patients with shoulder pain. *Arthritis Rheum* 2004; **50**: 3751-3761 [PMID: 15593187 DOI: 10.1002/art.20668]

2 **Riley G**. The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology* (Oxford) 2004; **43**: 131-142 [PMID: 12867575 DOI: 10.1093/rheumatology/keg448]

3 **Yamaguchi K**, Ditsios K, Middleton WD, Hildebolt CF, Galatz LM, Teefey SA. The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders. *J Bone Joint Surg Am* 2006; **88**: 1699-1704 [PMID: 16882890 DOI: 10.1016/j.jse.2010.07.017]

4 **Harryman DT**, Hettrich CM, Smith KL, Campbell B, Sidles JA, Matsen FA. A prospective multipractice investigation of patients with full-thickness rotator cuff tears: the importance of comorbidities, practice, and other covariables on self-assessed shoulder function and health status. *J Bone Joint Surg Am* 2003; **85-A**: 690-696 [PMID: 12672846]

5 **Yoo JH**, Cho NS, Rhee YG. Effect of postoperative repair integrity on health-related quality of life after rotator cuff repair: healed versus retear group. *Am J Sports Med* 2013; **41**: 2637-2644 [PMID: 23942286 DOI: 10.1177/0363546513499152]

6 **Kim HM**, Caldwell JM, Buza JA, Fink LA, Ahmad CS, Bigliani LU, Levine WN. Factors affecting satisfaction and shoulder function in patients with a recurrent rotator cuff tear. *J Bone Joint Surg Am* 2014; **96**: 106-112 [PMID: 24430409 DOI: 10.2106/JBJS.L.01649]

7 **Namdari S**, Donegan RP, Chamberlain AM, Galatz LM, Yamaguchi K, Keener JD. Factors affecting outcome after structural failure of repaired rotator cuff tears. *J Bone Joint Surg Am* 2014; **96**: 99-105 [PMID: 24430408 DOI: 10.2106/JBJS.M.00551]

8 **Park MC**, ElAttrache NS, Tibone JE, Ahmad CS, Jun BJ, Lee TQ. Part I: Footprint contact characteristics for a transosseous-equivalent rotator cuff repair technique compared with a double-row repair technique. *J Shoulder Elbow Surg* 2007; **16**: 461-468 [PMID: 17321161 DOI: 10.1016/j.jse.2006.09.010]

9 **Duquin TR**, Buyea C, Bisson LJ. Which method of rotator cuff repair leads to the highest rate of structural healing? A systematic review. *Am J Sports Med* 2010; **38**: 835-841 [PMID: 20357403 DOI: 10.1177/0363546509359679]

10 **Bishop J**, Klepps S, Lo IK, Bird J, Gladstone JN, Flatow EL. Cuff integrity after arthroscopic versus open rotator cuff repair: a prospective study. *J Shoulder Elbow Surg* 2006; **15**: 290-299 [PMID: 16679227 DOI: 10.1016/j.jse.2005.09.017]

11 **Tashjian RZ**, Hollins AM, Kim HM, Teefey SA, Middleton WD, Steger-May K, Galatz LM, Yamaguchi K. Factors affecting healing rates after arthroscopic double-row rotator cuff repair. *Am J Sports Med* 2010; **38**: 2435-2442 [PMID: 21030564 DOI: 10.1177/0363546510382835]

12 **Kobayashi M**, Itoi E, Minagawa H, Miyakoshi N, Takahashi S, Tuoheti Y, Okada K, Shimada Y. Expression of growth factors in the early phase of supraspinatus tendon healing in rabbits. *J Shoulder Elbow Surg* 2006; **15**: 371-377 [PMID: 16679241 DOI: 10.1016/j.jse.2005.09.003]

13 **Galatz LM**, Sandell LJ, Rothermich SY, Das R, Mastny A, Havlioglu N, Silva MJ, Thomopoulos S. Characteristics of the rat supraspinatus tendon during tendon-to-bone healing after acute injury. *J Orthop Res* 2006; **24**: 541-550 [PMID: 16456829 DOI: 10.1002/jor.20067]

14 **Kovacevic D**, Rodeo SA. Biological augmentation of rotator cuff tendon repair. *Clin Orthop Relat Res* 2008; **466**: 622-633 [PMID: 18264850 DOI: 10.1007/s11999-007-0112-4]

15 **Dines JS**, Grande DA, Dines DM. Tissue engineering and rotator cuff tendon healing. *J Shoulder Elbow Surg* 2007; **16**: S204-S207 [PMID: 17524676 DOI: 10.1016/j.jse.2007.03.004]

16 **Rodeo SA**. Biologic augmentation of rotator cuff tendon repair. *J Shoulder Elbow Surg* 2007; **16**: S191-S197 [PMID: 17574875 DOI: 10.1016/j.jse.2007.03.012]

17 **Murray DH**, Kubiak EN, Jazrawi LM, Araghi A, Kummer F, Loebenberg MI, Zuckerman JD. The effect of cartilage-derived morphogenetic protein 2 on initial healing of a rotator cuff defect in a rat model. *J Shoulder Elbow Surg* 2007; **16**: 251-254 [PMID: 17113320 DOI: 10.1016/j.jse.2006.07.002]

18 **Maniscalco P**, Gambera D, Lunati A, Vox G, Fossombroni V, Beretta R, Crainz E. The "Cascade" membrane: a new PRP device for tendon ruptures. Description and case report on rotator cuff tendon. *Acta Biomed* 2008; **79**: 223-226 [PMID: 19260383]

19 **Derwin KA**, Codsi MJ, Milks RA, Baker AR, McCarron JA, Iannotti JP. Rotator cuff repair augmentation in a canine model with use of a woven poly-L-lactide device. *J Bone Joint Surg Am* 2009; **91**: 1159-1171 [PMID: 19411465 DOI: 10.2106/JBJS.H.00775]

20 **Gulotta LV**, Kovacevic D, Ehteshami JR, Dagher E, Packer JD, Rodeo SA. Application of bone marrow-derived mesenchymal stem cells in a rotator cuff repair model. *Am J Sports Med* 2009; **37**: 2126-2133 [PMID: 19684297 DOI: 10.1177/0363546509339582]

21 **Isaac C**, Gharaibeh B, Witt M, Wright VJ, Huard J. Biologic approaches to enhance rotator cuff healing after injury. *J Shoulder Elbow Surg* 2012; **21**: 181-190 [PMID: 22244061 DOI: 10.1016/j.jse.2011.10.004]

22 **Milano G**, Saccomanno MF, Careri S, Taccardo G, De Vitis R, Fabbriciani C. Efficacy of marrow-stimulating technique in arthroscopic rotator cuff repair: a prospective randomized study. *Arthroscopy* 2013; **29**: 802-810 [PMID: 23522987 DOI: 10.1016/j.arthro.2013.01.019]

23 **Lopez-Vidriero E**, Goulding KA, Simon DA, Sanchez M, Johnson DH. The use of platelet-rich plasma in arthroscopy and sports medicine: optimizing the healing environment. *Arthroscopy* 2010; **26**: 269-278 [PMID: 20141991 DOI: 10.1016/j.arthro.2009.11.015]

24 **Sánchez Márquez JM,** Martínez Díez JM, Barco R, Antuña S. Functional results after arthroscopic repair of massive rotator cuff tears; influence of the application platelet-rich plasma combined with fibrin. *Revista Española De Cirugía Ortopédica Y Traumatología* (English Edition) 2011; **55**: 282-287

25 **Randelli P**, Arrigoni P, Ragone V, Aliprandi A, Cabitza P. Platelet rich plasma in arthroscopic rotator cuff repair: a prospective RCT study, 2-year follow-up. *J Shoulder Elbow Surg* 2011; **20**: 518-528 [PMID: 21570659 DOI: 10.1016/j.jse.2011.02.008]

26 **Nixon AJ**, Watts AE, Schnabel LV. Cell- and gene-based approaches to tendon regeneration. *J Shoulder Elbow Surg* 2012; **21**: 278-294 [PMID: 22244071 DOI: 10.1016/j.jse.2011.11.015]

27 **Lui P**, Zhang P, Chan K, Qin L. Biology and augmentation of tendon-bone insertion repair. *J Orthop Surg Res* 2010; **5**: 59 [PMID: 20727196 DOI: 10.1186/1749-799X-5-59]

28 **Thomopoulos S**, Genin GM, Galatz LM. The development and morphogenesis of the tendon-to-bone insertion - what development can teach us about healing. *J Musculoskelet Neuronal Interact* 2010; **10**: 35-45 [PMID: 20190378]

29 **Carpenter JE**, Thomopoulos S, Flanagan CL, DeBano CM, Soslowsky LJ. Rotator cuff defect healing: a biomechanical and histologic analysis in an animal model. *J Shoulder Elbow Surg* 1998; **7**: 599-605 [PMID: 9883420]

30 **Diaz Heredia J,** Ruiz Ibán M. A, Martinez Botas M, Aranda Romero F, Moros Marco S, Gonzalez Lizan F, Martin Gamero FJP. Respuesta a la lesión del manguito de los rotadores:Variación en la expresión de factores de crecimiento durante las primeras 8 semanas poslesión. Estudio experimental en ratas. *Cuadernos De Artroscopia* 2012; **19**: 31-38

31 **Cowin AJ**, Holmes TM, Brosnan P, Ferguson MW. Expression of TGF-beta and its receptors in murine fetal and adult dermal wounds. *Eur J Dermatol* 2001; **11**: 424-431 [PMID: 11525949]

32 **Galatz L**, Rothermich S, VanderPloeg K, Petersen B, Sandell L, Thomopoulos S. Development of the supraspinatus tendon-to-bone insertion: localized expression of extracellular matrix and growth factor genes. *J Orthop Res* 2007; **25**: 1621-1628 [PMID: 17600822 DOI: 10.1002/jor.20441]

33 **Akiyama H**, Kim JE, Nakashima K, Balmes G, Iwai N, Deng JM, Zhang Z, Martin JF, Behringer RR, Nakamura T, de Crombrugghe B. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci USA* 2005; **102**: 14665-14670 [PMID: 16203988 DOI: 10.1073/pnas.0504750102]

34 **Chen X**, Yin Z, Chen JL, Shen WL, Liu HH, Tang QM, Fang Z, Lu LR, Ji J, Ouyang HW. Force and scleraxis synergistically promote the commitment of human ES cells derived MSCs to tenocytes. *Sci Rep* 2012; **2**: 977 [PMID: 23243495 DOI: 10.1038/srep00977]

35 **Garofalo R**, Cesari E, Vinci E, Castagna A. Role of metalloproteinases in rotator cuff tear. *Sports Med Arthrosc* 2011; **19**: 207-212 [PMID: 21822103 DOI: 10.1097/JSA.0b013e318227b07b]

36 **Del Buono A**, Oliva F, Longo UG, Rodeo SA, Orchard J, Denaro V, Maffulli N. Metalloproteases and rotator cuff disease. *J Shoulder Elbow Surg* 2012; **21**: 200-208 [PMID: 22244063 DOI: 10.1016/j.jse.2011.10.020]

37 **Castagna A**, Cesari E, Garofalo R, Gigante A, Conti M, Markopoulos N, Maffulli N. Matrix metalloproteases and their inhibitors are altered in torn rotator cuff tendons, but also in the macroscopically and histologically intact portion of those tendons. *Muscles Ligaments Tendons J* 2013; **3**: 132-138 [PMID: 24367772]

38 **Bedi A**, Fox AJ, Kovacevic D, Deng XH, Warren RF, Rodeo SA. Doxycycline-mediated inhibition of matrix metalloproteinases improves healing after rotator cuff repair. *Am J Sports Med* 2010; **38**: 308-317 [PMID: 19826139 DOI: 10.1177/0363546509347366]

39 **Dean BJ**, Franklin SL, Carr AJ. A systematic review of the histological and molecular changes in rotator cuff disease. *Bone Joint Res* 2012; **1**: 158-166 [PMID: 23610686 DOI: 10.1302/2046-3758.17.2000115]

40 **Kovacevic D**, Fox AJ, Bedi A, Ying L, Deng XH, Warren RF, Rodeo SA. Calcium-phosphate matrix with or without TGF-β3 improves tendon-bone healing after rotator cuff repair. *Am J Sports Med* 2011; **39**: 811-819 [PMID: 21406666 DOI: 10.1177/0363546511399378]

41 **Chung SW**, Song BW, Kim YH, Park KU, Oh JH. Effect of platelet-rich plasma and porcine dermal collagen graft augmentation for rotator cuff healing in a rabbit model. *Am J Sports Med* 2013; **41**: 2909-2918 [PMID: 24047553 DOI: 10.1177/0363546513503810]

42 **Seeherman HJ**, Archambault JM, Rodeo SA, Turner AS, Zekas L, D'Augusta D, Li XJ, Smith E, Wozney JM. rhBMP-12 accelerates healing of rotator cuff repairs in a sheep model. *J Bone Joint Surg Am* 2008; **90**: 2206-2219 [PMID: 18829919 DOI: 10.2106/JBJS.G.00742]

43 **Chhabra A**, Tsou D, Clark RT, Gaschen V, Hunziker EB, Mikic B. GDF-5 deficiency in mice delays Achilles tendon healing. *J Orthop Res* 2003; **21**: 826-835 [PMID: 12919870 DOI: 10.1016/S0736-0266(03)00049-4]

44 **Thomopoulos S**, Harwood FL, Silva MJ, Amiel D, Gelberman RH. Effect of several growth factors on canine flexor tendon fibroblast proliferation and collagen synthesis in vitro. *J Hand Surg Am* 2005; **30**: 441-447 [PMID: 15925149 DOI: 10.1016/j.jhsa.2004.12.006]

45 **Chaudhury S**, Carr AJ. Lessons we can learn from gene expression patterns in rotator cuff tears and tendinopathies. *J Shoulder Elbow Surg* 2012; **21**: 191-199 [PMID: 22244062 DOI: 10.1016/

j.jse.2011.10.022]
46 **Chahal J**, Van Thiel GS, Mall N, Heard W, Bach BR, Cole BJ, Nicholson GP, Verma NN, Whelan DB, Romeo AA. The role of platelet-rich plasma in arthroscopic rotator cuff repair: a systematic review with quantitative synthesis. *Arthroscopy* 2012; **28**: 1718-1727 [PMID: 22694941 DOI: 10.1016/j.arthro.2012.03.007]
47 **Sánchez M**, Anitua E, Orive G, Padilla S. A biological approach to orthopaedic surgery: are they lost in translation? *Arthroscopy* 2013; **29**: 969-970 [PMID: 23726104 DOI: 10.1016/j.arthro.2013.02.017]
48 **Moraes VY**, Lenza M, Tamaoki MJ, Faloppa F, Belloti JC. Platelet-rich therapies for musculoskeletal soft tissue injuries. *Cochrane Database Syst Rev* 2013; **12**: CD010071
49 **Ahmad Z**, Brooks R, Kang SN, Weaver H, Nunney I, Tytherleigh-Strong G, Rushton N. The effect of platelet-rich plasma on clinical outcomes in lateral epicondylitis. *Arthroscopy* 2013; **29**: 1851-1862 [PMID: 24060428 DOI: 10.1016/j.arthro.2013.07.272]
50 **Raeissadat SA**, Sedighipour L, Rayegani SM, Bahrami MH, Bayat M, Rahimi R. Effect of Platelet-Rich Plasma (PRP) versus Autologous Whole Blood on Pain and Function Improvement in Tennis Elbow: A Randomized Clinical Trial. *Pain Res Treat* 2014; **2014**: 191525 [PMID: 24579044 DOI: 10.1155/2014/191525]
51 **Ruiz-Moneo P**, Molano-Muñoz J, Prieto E, Algorta J. Plasma rich in growth factors in arthroscopic rotator cuff repair: a randomized, double-blind, controlled clinical trial. *Arthroscopy* 2013; **29**: 2-9 [PMID: 23276410 DOI: 10.1016/j.arthro.2012.08.014]
52 **Gumina S**, Campagna V, Ferrazza G, Giannicola G, Fratalocchi F, Milani A, Postacchini F. Use of platelet-leukocyte membrane in arthroscopic repair of large rotator cuff tears: a prospective randomized study. *J Bone Joint Surg Am* 2012; **94**: 1345-1352 [PMID: 22854988 DOI: 10.2106/JBJS.K.00394]
53 **Anitua E**, Sánchez M, Orive G. The importance of understanding what is platelet-rich growth factor (PRGF) and what is not. *J Shoulder Elbow Surg* 2011; **20**: e23-e24; author reply e24 [PMID: 21050777 DOI: 10.1016/j.jse.2010.07.005]
54 **Lendeckel S**, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J Craniomaxillofac Surg* 2004; **32**: 370-373 [PMID: 15555520 DOI: 10.1016/j.jcms.2004.06.002]
55 **Ruiz-Ibán MÁ**, Díaz-Heredia J, García-Gómez I, Gonzalez-Lizán F, Elías-Martín E, Abraira V. The effect of the addition of adipose-derived mesenchymal stem cells to a meniscal repair in the avascular zone: an experimental study in rabbits. *Arthroscopy* 2011; **27**: 1688-1696 [PMID: 22001734 DOI: 10.1016/j.arthro.2011.06.041]
56 **Mazzocca AD**, McCarthy MB, Chowaniec D, Cote MP, Judson CH, Apostolakos J, Solovyova O, Beitzel K, Arciero RA. Bone marrow-derived mesenchymal stem cells obtained during arthroscopic rotator cuff repair surgery show potential for tendon cell differentiation after treatment with insulin. *Arthroscopy* 2011; **27**: 1459-1471 [PMID: 21978434 DOI: 10.1016/j.arthro.2011.06.029]
57 **García-Gómez I**, Elvira G, Zapata AG, Lamana ML, Ramírez M, Castro JG, Arranz MG, Vicente A, Bueren J, García-Olmo D. Mesenchymal stem cells: biological properties and clinical applications. *Expert Opin Biol Ther* 2010; **10**: 1453-1468 [PMID: 20831449 DOI: 10.1517/14712598.2010.519333]
58 **Lim JK**, Hui J, Li L, Thambyah A, Goh J, Lee EH. Enhancement of tendon graft osteointegration using mesenchymal stem cells in a rabbit model of anterior cruciate ligament reconstruction. *Arthroscopy* 2004; **20**: 899-910 [PMID: 15525922 DOI: 10.1016/j.arthro.2004.06.035]
59 **Ogawa R**, Mizuno H, Hyakusoku H, Watanabe A, Migita M, Shimada T. Chondrogenic and osteogenic differentiation of adipose-derived stem cells isolated from GFP transgenic mice. *J Nippon Med Sch* 2004; **71**: 240-241 [PMID: 15329483]
60 **Seo MJ**, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem Biophys Res Commun* 2005; **328**: 258-264 [PMID: 15670778 DOI: 10.1016/j.bbrc.2004.12.158]
61 **Brzoska M**, Geiger H, Gauer S, Baer P. Epithelial differentiation of human adipose tissue-derived adult stem cells. *Biochem Biophys Res Commun* 2005; **330**: 142-150 [PMID: 15781243 DOI: 10.1016/j.bbrc.2005.02.141]
62 **Kida Y**, Morihara T, Matsuda K, Kajikawa Y, Tachiiri H, Iwata Y, Sawamura K, Yoshida A, Oshima Y, Ikeda T, Fujiwara H, Kawata M, Kubo T. Bone marrow-derived cells from the footprint infiltrate into the repaired rotator cuff. *J Shoulder Elbow Surg* 2013; **22**: 197-205 [PMID: 22543003 DOI: 10.1016/j.jse.2012.02.007]
63 **Gulotta LV**, Kovacevic D, Montgomery S, Ehteshami JR, Packer JD, Rodeo SA. Stem cells genetically modified with the developmental gene MT1-MMP improve regeneration of the supraspinatus tendon-to-bone insertion site. *Am J Sports Med* 2010; **38**: 1429-1437 [PMID: 20400753 DOI: 10.1177/0363546510361235]
64 **Gulotta LV**, Kovacevic D, Packer JD, Ehteshami JR, Rodeo SA. Adenoviral-mediated gene transfer of human bone morphogenetic protein-13 does not improve rotator cuff healing in a rat model. *Am J Sports Med* 2011; **39**: 180-187 [PMID: 20956264 DOI: 10.1177/0363546510379339]
65 **Gulotta LV**, Kovacevic D, Packer JD, Deng XH, Rodeo SA. Bone marrow-derived mesenchymal stem cells transduced with scleraxis improve rotator cuff healing in a rat model. *Am J Sports Med* 2011; **39**: 1282-1289 [PMID: 21335341 DOI: 10.1177/0363546510395485]
66 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952 DOI: 10.1091/mbc.E02/02/0105]
67 **Zuk PA**, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; **7**: 211-228 [PMID: 11304456 DOI: 10.1089/107632701300062859]
68 **Gimble JM**, Grayson W, Guilak F, Lopez MJ, Vunjak-Novakovic G. Adipose tissue as a stem cell source for musculoskeletal regeneration. *Front Biosci* (Schol Ed) 2011; **3**: 69-81 [PMID: 21196358]
69 **Bacou F**, el Andalousi RB, Daussin PA, Micallef JP, Levin JM, Chammas M, Casteilla L, Reyne Y, Nouguès J. Transplantation of adipose tissue-derived stromal cells increases mass and functional capacity of damaged skeletal muscle. *Cell Transplant* 2004; **13**: 103-111 [PMID: 15129756]
70 **Mizuno H**. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. *J Nippon Med Sch* 2009; **76**: 56-66 [PMID: 19443990 DOI: 10.1159/000110805]
71 **Oh JH**, Chung SW, Kim SH, Chung JY, Kim JY. 2013 Neer Award: Effect of the adipose-derived stem cell for the improvement of fatty degeneration and rotator cuff healing in rabbit model. *J Shoulder Elbow Surg* 2014; **23**: 445-455 [PMID: 24129058 DOI: 10.1016/j.jse.2013.07.054]
72 **Gharaibeh B**, Lu A, Tebbets J, Zheng B, Feduska J, Crisan M, Péault B, Cummins J, Huard J. Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. *Nat Protoc* 2008; **3**: 1501-1509 [PMID: 18772878 DOI: 10.1038/nprot.2008.142]
73 **Pelinkovic D**, Lee JY, Engelhardt M, Rodosky M, Cummins J, Fu FH, Huard J. Muscle cell-mediated gene delivery to the rotator cuff. *Tissue Eng* 2003; **9**: 143-151 [PMID: 12625963 DOI: 10.1089/107632703762687627]
74 **Shen W**, Chen J, Yin Z, Chen X, Liu H, Heng BC, Chen W, Ouyang HW. Allogenous tendon stem/progenitor cells in silk scaffold for functional shoulder repair. *Cell Transplant* 2012; **21**: 943-958 [PMID: 22405331 DOI: 10.3727/096368911X627453]
75 **Beitzel K**, McCarthy MB, Cote MP, Russell RP, Apostolakos J, Ramos DM, Kumbar SG, Imhoff AB, Arciero RA, Mazzocca AD. Properties of biologic scaffolds and their response to mesenchymal stem cells. *Arthroscopy* 2014; **30**: 289-298 [PMID: 24581253 DOI: 10.1016/j.arthro.2013.11.020]
76 **Gimble JM**, Bunnell BA, Guilak F. Human adipose-derived cells: an update on the transition to clinical translation. *Regen Med* 2012; **7**: 225-235 [PMID: 22397611 DOI: 10.2217/rme.11.119]
77 **Beitzel K**, McCarthy MB, Cote MP, Durant TJ, Chowaniec DM, Solovyova O, Russell RP, Arciero RA, Mazzocca AD. Comparison of mesenchymal stem cells (osteoprogenitors) harvested from proximal humerus and distal femur during arthroscopic surgery.

*Arthroscopy* 2013; **29**: 301-308 [PMID: 23290182 DOI: 10.1016/j.arthro.2012.08.021]

78 **Tsai CC**, Huang TF, Ma HL, Chiang ER, Hung SC. Isolation of mesenchymal stem cells from shoulder rotator cuff: a potential source for muscle and tendon repair. *Cell Transplant* 2013; **22**: 413-422 [PMID: 23006509 DOI: 10.3727/096368912X656090]

79 **Randelli P**, Conforti E, Piccoli M, Ragone V, Creo P, Cirillo F, Masuzzo P, Tringali C, Cabitza P, Tettamanti G, Gagliano N, Anastasia L. Isolation and characterization of 2 new human rotator cuff and long head of biceps tendon cells possessing stem cell-like self-renewal and multipotential differentiation capacity. *Am J Sports Med* 2013; **41**: 1653-1664 [PMID: 23393078 DOI: 10.1177/0363546512473572]

80 **Utsunomiya H**, Uchida S, Sekiya I, Sakai A, Moridera K, Nakamura T. Isolation and characterization of human mesenchymal stem cells derived from shoulder tissues involved in rotator cuff tears. *Am J Sports Med* 2013; **41**: 657-668 [PMID: 23371475 DOI: 10.1177/0363546512473269]

81 **Ellera Gomes JL**, da Silva RC, Silla LM, Abreu MR, Pellanda R. Conventional rotator cuff repair complemented by the aid of mononuclear autologous stem cells. *Knee Surg Sports Traumatol Arthrosc* 2012; **20**: 373-377 [PMID: 21773831 DOI: 10.1007/s00167-011-1607-9]

82 **Gimble J**, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 2003; **5**: 362-369 [PMID: 14578098 DOI: 10.1080/14653240310003026]

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REVIEW

# Epigenetic regulation of stemness maintenance in the neurogenic niches

Raquel Montalbán-Loro, Ana Domingo-Muelas, Alexandra Bizy, Sacri R Ferrón

Raquel Montalbán-Loro, Ana Domingo-Muelas, Alexandra Bizy, Sacri R Ferrón, Departamento de Biología Celular, Facultad de Biología, Universidad de Valencia, 46100 Burjassot, Spain
Author contributions: Montalbán-Loro R, Domingo-Muelas A, Bizy A and Ferrón SR all contributed to this paper.
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Correspondence to: Sacri R Ferrón, SR PhD, Departamento de Biología Celular, Facultad de Biología, Universidad de Valencia, C/ Dr. Moliner, 50, 46100 Burjassot, Spain. sacramento.rodriguez@uv.es
Telephone: +34-963-543784
Fax: +34-963-543404
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## Abstract

In the adult mouse brain, the subventricular zone lining the lateral ventricles and the subgranular zone in the dentate gyrus of the hippocampus are two zones that contain neural stem cells (NSCs) with the capacity to give rise to neurons and glia during the entire life of the animal. Spatial and temporal regulation of gene expression in the NSCs population is established and maintained by the coordinated interaction between transcription factors and epigenetic regulators which control stem cell fate. Epigenetic mechanisms are heritable alterations in genome function that do not involve changes in DNA sequence itself but that modulate gene expression, acting as mediators between the environment and the genome. At the molecular level, those epigenetic mechanisms comprise chemical modifications of DNA such as methylation, hydroxymethylation and histone modifications needed for the maintenance of NSC identity. Genomic imprinting is another normal epigenetic process leading to parental-specific expression of a gene, known to be implicated in the control of gene dosage in the neurogenic niches. The generation of induced pluripotent stem cells from NSCs by expression of defined transcription factors, provide key insights into fundamental principles of stem cell biology. Epigenetic modifications can also occur during reprogramming of NSCs to pluripotency and a better understanding of this process will help to elucidate the mechanisms required for stem cell maintenance. This review takes advantage of recent studies from the epigenetic field to report knowledge regarding the mechanisms of stemness maintenance of neural stem cells in the neurogenic niches.

**Key words:** Neurogenesis; Neural stem cell; Epigenetics; Gene expression regulation; Chromatin modifications; DNA methylation

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**Core tip:** Neural stem cells (NSCs) are capable of extensive self-renewal while preserving the ability to generate cell progeny that can differentiate into different cell types from the nervous system. Intrinsic mediators as well as extrinsic cues provided by the neurogenic niche (microenvironment where NSCs reside *in vivo*) are important for stem cell self-renewal and differentiation. Epigenetic changes, including alterations in DNA methylation, histone modifications and imprinting alter

the way a gene interacts with the cell transcribing machinery, turning genes "on" or "off". These heritable changes must be reversible and context-dependent being responsible of stem cell plasticity.

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## NEURAL STEM CELLS AND THE NEUROGENIC NICHES

Adult stem cells have the ability to divide, self-renew and generate functional differentiated cells that replace lost cells throughout an organism's lifetime. The existence of adult stem cells was first described in tissues with high proliferation rates, such as the hematopoietic system and the intestine. Since then, stem cells have been found in almost all adult tissues including the nervous system[1]. In the adult mouse brain two main regions continue to generate new neurons throughout adulthood: the subventricular zone (SVZ) in the walls of the lateral ventricles[2] (Figure 1A-C) and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus[3] (Figure 1D-F). Adult neurogenesis is supported by multipotent neural stem cells (NSCs) deriving from embryonic radial-glia and thus expressing astroglial characteristics[4,5]. Astrocytic-like stem cells are relatively quiescent and can be identified by the expression of the glial fibrillary acidic protein (GFAP), the stemness-related transcription factor Sox2 [Sex determining region Y (SRY)-box 2], and the neural progenitor marker Nestin[2,6,7]. Moreover, their slow division rate can be detected by the label retention of thymidine analogs incorporated during DNA replication[6,8,9]. NSCs can also be isolated from their natural niche and cultured *in vitro* in the presence of the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) mitogens. In culture, NSCs form free-floating aggregates called "*neurospheres*" (Figure 1C). Self-renewal and multipotency characteristics of NSCs are assessed *in vitro* by clonal analysis in which single cells give rise to neurospheres[10,11] (Figure 1C).

### *SVZ and the olfactory bulb system*

The SVZ is located lining the walls of the lateral ventricles and constitutes a complex microenvironment or niche in which proliferation and self-renewal of NSCs are strongly regulated by multiple extracellular factors such as EGF, bFGF, bone morphogenetic protein and pigment epithelium derived factor[12-14]. This significant extrinsic signaling is possible because of the special cytoarchitecture of the niche that allows NSCs to be in direct contact with the cerebrospinal fluid (CSF) produced by the choroid plexus in the ventricles, with the vasculature and with other cells from the niche like astrocytes or microglia[15,16]. Subventricular NSCs (also known as type B1 cells) present a radial glia-like morphology, with an apical primary cilium contacting the ventricular lumen and a basal process reaching the basal lamina and the vascular structures[17,18] (Figure 1A). The walls of the lateral ventricles show a typical organization where the small apical process of one or more type B1 cells are surrounded by a rosette of epithelial ependymal cells that form structures known as pinwheels at the surface[19]. There is another astrocyte-like type B cell that is more frequently located close to the underlying striatal parenchyma known as type B2 cells[20]. When activated, these slowly dividing NSCs give rise to fast cycling cells called transit-amplifying progenitors (TAP or type C cells). TAP cells contribute to reducing the number of cell division rounds that NSCs have to undergo to preserve their genome integrity. Mash1-positive type C cells generate neuroblasts (type A cells) that migrate along the rostral migratory stream (RMS) into the olfactory bulb (OB) where they differentiate and integrate into interneurons (Figure 1B). These chains of polysialylated neural cell adhesion molecule (PSA-NCAM) and DCX (doublecortin) positive neuroblasts reach the core of the OB, where they detach from the RMS and migrate radially into the granular and periglomerular layers[21-23]. These immature neurons then integrate and differentiate into inhibitory interneurons, playing an important role in rodent olfaction. In addition of being a neurogenic region, the SVZ can serve as a niche of oligodendrocytes although generated in much lower numbers than neuroblasts. Thereby, Olig2-positive transit amplifying cells give rise to oligodendroblasts that migrate to the corpus callosum and striatum while tightly associated with blood vessels[24], where they differentiate into myelinating and nonmyelinating oligodendrocytes[25].

### *SGZ of the hippocampus*

Along with the SVZ, the subgranular zone in the dentate gyrus of the hippocampus constitutes the other main neurogenic niche in the adult mouse brain[26-28]. The SGZ is also a complex microenvironment in which the vasculature plays an important role. Dividing stem cells in the SGZ are in close proximity to an extensive network of interconnected blood vessels and parenchymal astrocytes that can regulate their proliferation and differentiation *via* paracrine signaling[29]. The SGZ is located between the granular layer and the hilus of the DG and the SGZ NSCs constitute a subpopulation of GFAP-positive cells that are analogous to subventricular type B1 cells[30]. In this region, two types of neural progenitors can be identified according to different expression of molecular markers and their morphologies[23] (Figure 1D and E). Type Ⅰ progenitors exhibit a radial process spanning the granule cell layer and arborizing profusely in

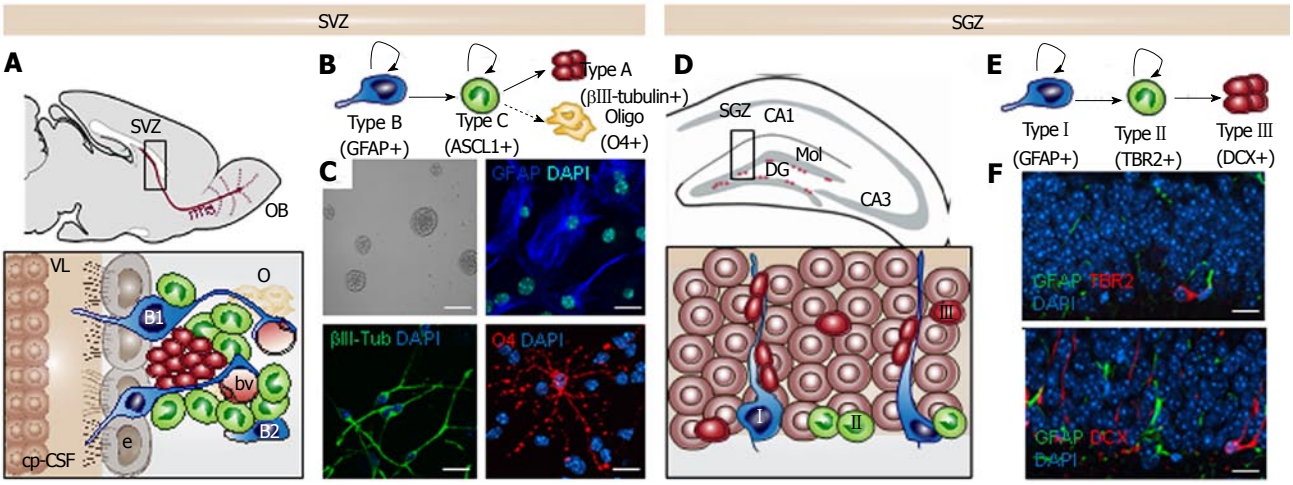


**Figure 1 The neurogenic niches in the adult murine mammalian brain.** A: Sagittal view showing the adult mouse subventricular zone (SVZ) and the migrating neuroblasts (red) reaching the olfactory bulb (OB) through the rostral migratory stream (rms). Enlarged view of SVZ: type B1 stem cells (blue) express the astrocyte marker glial fibrillary acidic protein (GFAP) and contact the ventricle with a thin process extended between the ependymal cells (e; gray); type B2 stem cells (blue) contacting the brain parenchyma; transit amplyfing progenitors (TAP) or type C cells (green) express the achaete-scute homolog 1 (ASCL1) transcription factor and give rise to type A cells (red) that migrate through the rostral migratory stream (rms). Dividing stem cells and their TAP progeny are tightly opposed to blood vessels (bv); B: Schematic drawing showing the lineage progression in the SVZ; C: SVZ neural stem cell (NSC) cultures in self-renewal (neurosphere formation) and differentiation. The astrocyte marker GFAP in blue, the neuronal marker βIII-tubulin in green and the oligodendrocyte marker O4 in red; The Choroid plexus-cerebrospinal fluid system (cp-CSF) is shown. D: Coronal view showing the adult mouse subgranular zone (SGZ) and the newborn neurons (red) being integrated in the granular cell layer (gr). Enlarged view of the dentate gyrus (DG): Type I stem cells (blue) are GFAP+ and show a radial single prolongation through the granular layer; Type II precursors give rise to neuronal lineage-restricted progenitors type III cells (red) that differentiate into neurons in the granular layer; E: Schematic drawing showing the lineage progression in the SGZ; F: Confocal images showing immunostaining in the DG for the astrocyte marker GFAP in green, for the progenitor precursor marker T-box brain protein 2 (TBR2) in red and for the neuronal precursor marker Doublecortin (DCX) in red. DAPI is used to stain DNA. Scale bar in C: Top left panel 100 μm, rest 10 μm; In f: 10 μm.

the molecular layer[27]. These cells express nestin, GFAP, and Sox2[31]. Type II hippocampal progenitors have short processes and contrary to type I cells, express TRB2 but not GFAP (Figure 1F). There is evidence suggesting that type II cells may derive from type I cells but a lineage relationship study is still lacking[31]. In the adult SGZ, precursors give rise by asymmetrical divisions to intermediate neuronal lineage-restricted progenitor cells and in a minor number, to glial lineage-restricted progenitor cells (both of them are GFAP-negative cells). Compared to the SVZ, few oligodendrocytes are generated in the SGZ. Type II cells generate in turn type III cells, which are neuronal precursors that express markers of immature migrating neurons, such DCX and PSA-NCAM (Figure 1F). These differentiated cells integrate neuronal circuits into the hippocampal CA3 region forming dendrites and spreading their axons[22]. In addition to the production of granular neurons, a low percentage of activated NSCs divide asymmetrically to give rise to astrocytes. The latter migrate into the hilus and the molecular layer where they lose their stem cell identity and cause the depletion of the pool of NSCs[32,33] thus explaining the possible decrease in hippocampal neurogenesis associated with ageing.

## EPIGENETIC REGULATION OF GENE EXPRESSION IN THE NEUROGENIC NICHES

Epigenetic is defined as the study of heritable alterations in genome function that do not involve changes in DNA sequence itself[34,35]. These epigenetic marks modulate gene expression either by directly altering the chromatin structure or by creating bindings sites for chromatin and transcription regulatory subunits. Two general classes of epigenetic regulation can be defined: covalent modifications to DNA and post-translational covalent modifications to the histones (H) around which the DNA is bound, influencing whether DNA is accessible or not for gene transcription[36,37] (Figure 2A). Moreover, the three-dimensional structure and arrangement of chromatin within the nucleus are both regulated by and contribute to the establishment and maintenance of epigenetic states[34]. These different classes of epigenetic modifications are intimately related, resulting in multiple layers of control allowing cells to maintain their identity over time[34,38]. Dysregulation of these mechanisms leads to new cellular phenotypes by causing altered gene expression without a change in genotype. In the neurogenic niches, epigenetic regulators and the associated transcription factors play an important role in the control and maintenance of NSC stemness.

### *DNA methylation*

DNA methylation involves the addition of a methyl group to the fifth carbon in the cytosine pyrimidine ring (Figure 2B). In most mammalian genes, CpG dinucleotides are methylated and concentrated in clusters called "CpG islands" which often have regulatory functions and tend to be found in the promoter and first exon regions of genes[39] where it promotes a closed chromatin structure and aids the prevention

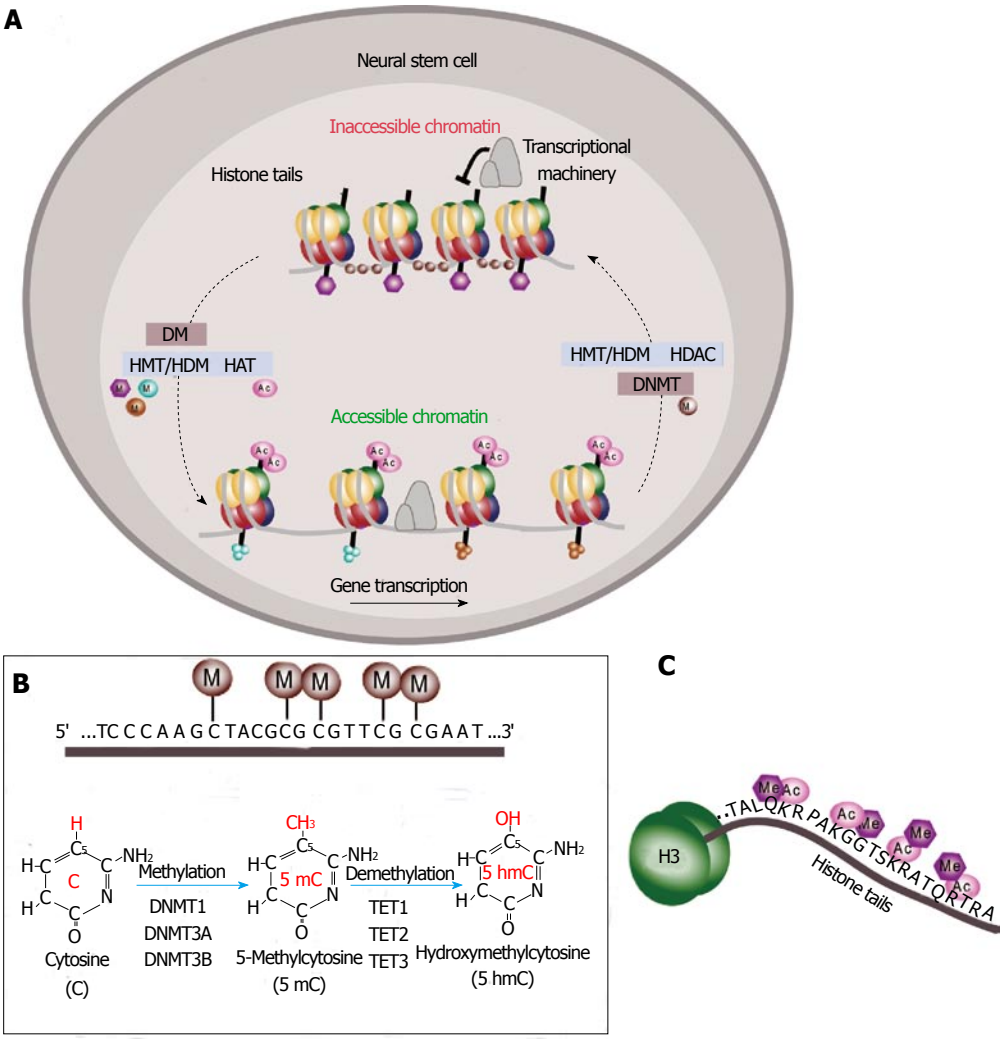


**Figure 2 Epigenetic regulation of gene expression.** A: Schematic of DNA methylation and histone modifications in neural stem cells (NSCs). DNA is compressed through interactions with histones and methyl groups (M) are added to cytosine-guanine (CpGs) dinucleotides in regulatory regions. Histone methylation reactions are catalyzed by histone methyltransferases (HMTs) and the reverse process is mediated by histone demethylases (HDMs). Histone acetylation is mediated by histone acetyltransferases (HATs) that leads to chromatin decondensation (accessible chromatin) and transcription activation. Histone deacetylases (HDACs) catalyze the reverse process inducing inactivation of transcription (inaccessible chromatin); B: Schematic of DNA methylation at the cytosine-guanine dinucleotides in gene regulatory regions. Methylation reactions are mediated by DNA methyltransferases (DNMTs) that transfer methyl groups (M) to the fifth position of the pyrimidine ring. This is a reversible process mediated by the ten-eleven translocation (TET) family of enzymes TET1, TET2 and TET3 dioxygenases that catalyze the conversion of the modified genomic base 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) playing a key role in active DNA demethylation; C: Schematic of the histone tail showing multiple sites for epigenetic modifications as acetylation (Ac) or methylation (Me).

of expression[34,40]. DNA methylation marks repress gene expression either by attracting DNA methyl-binding domain proteins (MBDs) such as methyl-CpG binding protein 2 (MeCP2) which recruit repressors and chromatin remodeling molecules to generate an inactive chromatin environment or by directly inhibiting transcription factor binding[41-43]. MBD proteins have been suggested to play a role in neurogenesis. For example, mice deficient in MBD1 show decreased neurogenesis and hippocampus-related behaviour defects. Indeed, *Mbd1*⁻deficient NSCs generate a reduced number of neurons when compared to wild-type cells, suggesting a role for MBD1 in neuronal fate commitment[44].

There are two types of methylation reactions both mediated by DNA methyltransferases (DNMTs). One is *de novo* methylation catalyzed by DNMT3a and DNMT3b, important for normal embryogenesis and development and responsible for the establishment of methylation patterns. The other type is maintenance methylation mediated by DNMT1 that effectively maintains CpG methylation upon DNA replication and provides the heritable "memory" of the methylation state of the parent cell[45,46]. DNMT1 is highly expressed in the embryonic, perinatal and adult CNS in both dividing neural progenitors and mature neurons where it maintains DNA methylation[47-49]. A lack of DNMT1 alters neuronal excitability and increases apoptosis in post-mitotic cortical neurons[50]. In support of this, mice deficient for *Dnmt1* specifically in neural progenitors at embryonic stages exhibit deficits in neuronal function and die postnatally, suggesting a requirement for methylation in brain development[51]. DNMT3a and DNMT3b are highly expressed in postnatal

NSCs and are required for neurogenesis and neuronal maturation[48,49,52,53]. Loss of DNMT3a results in gene silencing[53] and depletion of DNMT3b leads to deficient neuronal differentiation *in vitro*[54].

DNA is hypomethylated in neural progenitor cells and methylation is progressively increased during lineage commitment[55]. The suppression of astrogliogenesis during neuronal specification is also associated with changes in DNA methylation[56,57]. This silencing is attenuated later in development resulting in the generation of astrocytes which correlates with the suppression of neurogenesis. Demethylation and expression of the genes coding for the astrocytic markers *Gfap* and the calcium binding protein *S100β* during astrocytic maturation, correlates with methylation and downregulation of neurogenic genes such as Neurogenin 1[58-60]. Activation of the *Gfap* promoter requires binding of the signal transducer and activator of transcription 3 (STAT3) to a consensus sequence. Early progenitors are refractory to astrocyte differentiation due to methylation of the STAT3 binding site. At later development stages, loss of STAT3-binding element methylation is associated with *Gfap* promoter activation[60,61]. A similar alteration in methylation pattern occurs at another STAT3 binding site in the *S100β* promoter[58].

### Hydroxymethylation

DNA methylation marks are reversible through both passive replication-dependent demethylation and active demethylation which probably involve the recently characterized 5-hydroxymethyl (5hmC) intermediate[62] (Figure 2B). In mammals, three members of the ten-eleven translocation (TET) family of enzymes have been identified: TET1, TET2 and TET3[63,64]. TET hydroxylases may catalyze active DNA demethylation by oxidation of 5mC to 5hmC[65-67] (Figure 2B). 5hmC is relatively abundant in mouse embryonic stem cells (ESCs), the early embryo and in adult brain[68,69]. In the brain, 5hmC is enriched at active genes, associated with the strong depletion of 5mC from these regions[70]. It has been proposed that TET enzymes in the blastocyst and ESCs are involved in pluripotency by maintaining the hypomethylated state of key regulatory regions[69,71]. Recent studies have also shown that TET1 is involved in the epigenetic regulation of neural progenitor cell proliferation in the adult hippocampus[72-74]. Mice lacking *Tet1* exhibit impaired hippocampal neurogenesis accompanied by poor learning and memory[72-74]. However, the full role and importance of hydroxymethylation in the brain remains to be elucidated.

### Histone modifications as regulators of adult neurogenesis

In eukaryotic cells, a histone octamer including two H2A-H2B dimers and a H3-H4 tetramer acts as a scaffold around which DNA is wrapped to form a nucleosome[75,76]. The interaction between histones and DNA is mediated by an N-terminal tail of histone proteins available for post-translational modifications that control the chromatin structure[75] (Figure 2C). These covalent modifications in the histone tails alter the interaction between adjacent nucleosomes and/or between histones and the DNA, changing the three-dimensional chromatin structure. Modifications in the body of histones have also been shown to alter chromatin structure influencing gene expression[77]. Histone modifications are divided into repressive and active marks according to how they correlate with levels of transcriptional activity. For example, histone acetylation of lysine residues of histones, catalyzed by histone acetyltransferases enhances the recruitment and activation of the transcriptional machinery and is generally associated with areas of active gene transcription[78]. However, histone deacetylases (HDACs) remove acetyl groups promoting the condensation of chromatin[79] (Figure 2A). HDAC1 is expressed by GFAP-positive cells within the SVZ whereas HDAC2 is found in migrating neuroblasts and in TAP cells within the SVZ[80]. Deletion of HDAC2 in the SVZ results in a defective neurogenesis to the OB[81] and neurospheres treated with class Ⅰ and Ⅱ HDAC inhibitors promotes neuronal differentiation[82] suggesting a role for this enzyme in neuronal fate determination. Furthermore, oligodendrocyte fate commitment is accompanied by a decrease in histone deacetylation at transcriptional repressors of oligodendrocytic differentiation such as *Sox11*[83] and at neuronal genes such as *Sox2*[84].

Histone methylation is associated with both active and silent chromatin and is catalyzed by histone methyltransferases (HMTs). Trimethylation of lysine (K)-27 and lysine 9 of histone H3 (H3K27me$^3$ and H3K9me$^3$) tends to associate with regions of inactive gene transcription, whereas H3K4, H3K36 and H3K79 methylations are associated with active transcription[85]. Histone demethylases (HDMs) also have a key role in regulating neural development[86]. During neural stem cell commitment, H3K27 methylation decreases in key developmental genes following downregulation of the HMT *Enhancer of Zest homolog 2* (EZH2) and upregulation of the HDM Jumonji domain containing-3 (JMJD3). Indeed, deletion of *Ezh2* in SGZ progenitor cells results in cell proliferation restriction leading to a reduced number of neurons that ultimately leads to impairment in spatial learning and memory[87]. Additionally, JMJD3 is upregulated in neuroblasts, and *Jmjd3* deletion targeted to SVZ NSCs in both developing and adult mice impairs neuronal differentiation. JMJD3 regulates neurogenic gene expression *via* interaction at not only promoter regions but also neurogenic enhancer elements such as Dlx2[88]. Moreover, H3K9me$^3$ is enriched in the adult murine SVZ and it has been recently shown that its repression in undifferentiated cells is engaged in the maintenance of cell type integrity in this neurogenic niche[89]. MLL1, another HMT that methylates H3K4, has been associated with the trithorax group of transcription factors. In mice where *Mll1* is knocked out in NSCs, neurogenesis is impaired.

*Mll1* is associated with the promoter of the homeobox transcription factor *Dlx2* and although loss of *Mll1* does not affect the methylation of H3K4, it does increase H3K27me$^3$ on the promoter indicating that *Mll1* is recruiting a H3K27 demethylase[90]. In summary, the above studies indicate that different chromatin modifiers have a critical role in adult neurogenesis[91].

### Genomic imprinting and control of gene dosage

Imprinted genes are expressed predominantly from one chromosome in a parental-origin dependent manner. While most genes are expressed from both alleles, imprinted genes are functionally monoallelic and are expressed from either the maternally or the paternally inherited chromosome[92]. In mammals, this affects around 100 genes that are found in clusters. Imprinting control regions (ICRs) regulate the parental allele-specific pattern of gene expression and have differentially methylated regions (DMRs) on the two parental chromosomes. ICRs can be divided into those which are methylated on the paternally inherited copy and those with maternally inherited methylation[93]. DMRs are also characterized by the asymmetrical accumulation of different histone modifications on the two parental chromosomes and the recent identification of a "tri-mark", comprising the trimethylation of H3K4 and H3K9 and the trimethylation of H4K20 at all known ICRs[94]. The majority of imprinted genes are expressed in the brain and several exhibit brain-specific imprinting. Their monoallelic expression makes these loci very vulnerable as mutation of the expressed allele can compromise expression and lead to severe developmental defects. For example, human congenital imprinting syndromes including Angelman syndrome and Prader-Willi syndrome are all characterized by neurological and behavioral impairments and learning difficulties[95]. Evidence is suggesting that selective regulation of imprinting is a normal mechanism of modulating gene dosage and is associated with the control of stem cell potential in the neurogenic niche. For instance, relaxation of imprinting of the gene for the atypical NOTCH ligand delta-like homologue 1 (*Dlk1*) usually expressed from the paternally inherited chromosome has been shown in the neural stem cells and niche astrocytes within the SVZ[96]. Notably, this selective absence of *Dlk1* imprinting is associated with acquisition of DNA methylation at the germline-derived imprinting control region[96]. *Igf2* is also an imprinted gene expressed only from the paternally-inherited allele although it is specifically biallelically expressed in postnatal human and mouse choroid plexus epithelium and leptomeninges[97,98]. Thus, CSF produced from the choroid plexus and blood vessels is a biallelic source of neurogenesis-promoting IGF2[99].

### Epigenetic changes during NSCs reprogramming to induced pluripotent stem cells

Epigenetic reprogramming consists in the transition from one cell type to another, permitted by the loss of the molecular characteristics of the cell of origin and the acquisition of an entirely new molecular identity without changing the genomic sequence[100]. Reprogramming involves changes in the transcriptome and chromatin state of the reprogrammed cell type to that of a pluripotent cell[101-103]. This implicates different levels of changes in DNA factor binding, transcription and chromatin state[103]. Since the discovery by Takahashi and Yamanaka in 2006 that the introduction of four transcription factors, *Oct3/4*, *Klf4*, *c-Myc*, *Sox2* (known as OKMS) could reprogram mouse embryonic and adult fibroblasts into induced pluripotent stem cells (iPSCs)[104], the field of reprogramming has considerably evolved and several studies have reported the use of sets of these transcription factors in various combinations to reprogram mouse and human somatic cells[105-108]. More recently, murine B lymphocytes, liver, stomach and pancreatic β-cells were showed to reprogram into iPSCs using the combination of factors OKMS[109-111]. In 2008, Eminli *et al*[112] reported the generation of iPSCs from murine NSCs by retroviral infection of the same combination of factors. Since neurosphere cultures express *Sox2* and *c-myc*, a considerable advance consisted in showing that they could be reprogrammed only with Oct4 and Klf4 at similar efficiency to the reprogramming rate of murine fibroblasts with the original four factors[112-114]. Finally, the forced expression of *Oct4* alone was shown sufficient to reprogram murine NSCs, albeit with a ten-fold lower efficiency than with two factors[113]. Because NSCs are originally closer to the pluripotency state than somatic cells and require fewer factors to be reprogrammed, they constitute a more simple and attractive system to study epigenetic mechanisms occurring during the acquisition of pluripotency. Importantly, iPSCs derived from human and murine NSCs exhibited markers of ESCs, showed demethylation of pluripotency genes, formed teratomas, and contributed to viable chimeras[112-114].

### Reprogramming factors and epigenetic mechanisms

Reprogramming of somatic cells is a stochastic event[115]. However, in NSCs, *Oct4* only seems sufficient to repress genes responsible for NSCs molecular identity and activate the pluripotency genes, suggesting that epigenetic of NSCs renders them easier to reprogram and that the combination of factors necessary for reprogramming is dependent on cellular context[112]. iPSCs have lower levels of methylation than somatic cells, suggesting that demethylation is an important chromatin feature to achieve pluripotency[116]. During reprogramming, it is stipulated that reprogramming factors interfere with methylation of the newly synthesized DNA by binding to specific promoters or enhancer regions leading to demethylation and activation of the pluripotency genes. In addition, active DNA demethylation mechanisms could be required for the reactivation of pluripotency genes[117]. Recent studies in NSCs have shown the importance of methy-

lation level in the context of reprogramming. Undifferentiated neurospheres highly express DNMT1 and contain methylated chromatin suggesting the role of methylation for the maintenance of the quiescent or undifferentiated state of NSCs[118]. It is then probable that NSC chromatin is dynamically remodelled and that DNA methylation modification is essential for reprogramming to a pluripotent state. For instance, histone methyltransferase G9a is responsible for the downregulation of Oct4 during NSC differentiation and its inhibition results in iPSC formation after overexpression of exogenous Klf4 and c-myc only[119]. In addition, interference with DNMT1 promotes iPSC formation, also supporting that DNA methylation is a feature limiting reprogramming to pluripotency[101]. All reprogramming techniques involve demethylation of the genome thus appearing as a crucial process for successfully achieving pluripotency[120,121].

### *Loss of epigenetic memory*

During reprogramming, NSCs downregulate specific genes like *Nestin* and progressively express the markers of pluripotency *Oct4*, *Nanog*, *Fgf4*, *Zfp42*[113,114]. In addition, efficiency and timing of reprogramming highly depends on the differentiation state of the initial cell type. Importantly, comparative studies with ESCs reported that efficiently reprogrammed iPSCs show transcriptional pattern and epigenetic marks highly similar to ESCs. For instance, Oct4 and Nanog promoters are demethylated and histones H3 lysine 4 (K4) and lysine 27 mostly exhibit patterns of trimethylation[101,106,122]. However, reprogramming of NSCs into iPSCs is often incomplete and leaves epigenetic marks including DNA methylation, chromatin modification and transcriptional regulation in the resulting iPSC genome[123,124] known as epigenetic "memory". Partially reprogrammed cell lines are characterized by an absence of complete downregulation of the exogenous reprogramming factors and partial demethylation and reactivation of pluripotency genes[101,104]. During reprogramming, somatic markers get progressively downregulated demonstrating the importance of silencing its differentiation program as a step towards pluripotency. Treatment of partially reprogrammed iPSCs with inhibitors of ERK1/2 and GSK3b signaling[125], induced genome demethylation of 30% explained by decreased levels of DNMT3a/b and their targeting factor DNMT3L[126-128]. The two inhibitors repress DNMT3A/B expression inducing demethylation of certain genomic regions in ESCs. Thus, DNA demethylation of the reprogrammed cell type as a way to remove epigenetic marks is important for complete reprogramming into iPSCs. Reprogrammed iPSCs often present the limitation of not being fully reprogrammed thus keeping epigenetic traces of the tissue of origin. Future generation of iPSCs without epigenetic memory is an important challenge in the field to ensure that differentiation decisions are not affected by events from the past[116].

## FUTURE PERSPECTIVES

Determining the mechanisms by which neural stem cells maintain self-renewal capacity and at the same time generate differentiated progeny is a central challenge in stem cell biology. Several recent studies have demonstrated that epigenetic gene regulation plays a crucial role in the control of stem cell behaviour. Epigenetic mechanisms include changes in chromatin structure that provides a way for coordinately activating or repressing genes during proliferation and differentiation. Extracellular signaling from the microenvironment or niche in which NSCs reside *in vivo* interacts with these diverse epigenetic mechanisms, thus regulating transcription factors and intracellular pathways. These changes in gene expression are often heritable and reversible, features that support stem cell plasticity such as the ability to dedifferentiate or become reprogrammed under certain conditions. Finally, aberrant epigenetic mechanisms are known to be involved in the development of many neurological diseases. Characterizing epigenetic changes associated with a particular neural pathology may be used as biomarkers of disease and the manipulation of those epigenetic mechanisms holds great promise as a potential therapeutic strategy.

## REFERENCES


1 **Li L**, Xie T. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol* 2005; **21**: 605-631 [PMID: 16212509 DOI: 10.1146/annurev.cellbio.21.012704.131525]

2 **Doetsch F**, García-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 1997; **17**: 5046-5061 [PMID: 9185542]

3 **Gage FH**, Kempermann G, Palmer TD, Peterson DA, Ray J. Multipotent progenitor cells in the adult dentate gyrus. *J Neurobiol* 1998; **36**: 249-266 [PMID: 9712308]

4 **Doetsch F**. The glial identity of neural stem cells. *Nat Neurosci* 2003; **6**: 1127-1134 [PMID: 14583753 DOI: 10.1038/nn1144]

5 **Merkle FT**, Tramontin AD, García-Verdugo JM, Alvarez-Buylla A. Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc Natl Acad Sci USA* 2004; **101**: 17528-17532 [PMID: 15574494 DOI: 10.1073/pnas.0407893101]

6 **Ferri AL**, Cavallaro M, Braida D, Di Cristofano A, Canta A, Vezzani A, Ottolenghi S, Pandolfi PP, Sala M, DeBiasi S, Nicolis SK. Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development* 2004; **131**: 3805-3819 [PMID: 15240551 DOI: 10.1242/dev.01204]

7 **Morshead CM**, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 1994; **13**: 1071-1082 [PMID: 7946346]

8 **Nam HS**, Benezra R. High levels of Id1 expression define B1 type adult neural stem cells. *Cell Stem Cell* 2009; **5**: 515-526 [PMID: 19896442 DOI: 10.1016/j.stem.2009.08.017]

9 **Ponti G**, Obernier K, Guinto C, Jose L, Bonfanti L, Alvarez-Buylla A. Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. *Proc Natl Acad Sci USA* 2013; **110**: E1045-E1054 [PMID: 23431204 DOI: 10.1073/pnas.1219563110]

10 **Ferron SR**, Andreu-Agullo C, Mira H, Sanchez P, Marques-Torrejon MA, Farinas I. A combined ex/in vivo assay to detect

effects of exogenously added factors in neural stem cells. *Nat Protoc* 2007; **2**: 849-859 [PMID: 17474182]
11 **Reynolds BA**, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; **255**: 1707-1710 [PMID: 1553558]
12 **Faigle R**, Song H. Signaling mechanisms regulating adult neural stem cells and neurogenesis. *Biochim Biophys Acta* 2013; **1830**: 2435-2448 [PMID: 22982587 DOI: 10.1016/j.bbagen.2012.09.002]
13 **Porlan E**, Perez-Villalba A, Delgado AC, Ferrón SR. Paracrine regulation of neural stem cells in the subependymal zone. *Arch Biochem Biophys* 2013; **534**: 11-19 [PMID: 23073070 DOI: 10.1016/j.abb.2012.10.001]
14 **Ramírez-Castillejo C**, Sánchez-Sánchez F, Andreu-Agulló C, Ferrón SR, Aroca-Aguilar JD, Sánchez P, Mira H, Escribano J, Fariñas I. Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. *Nat Neurosci* 2006; **9**: 331-339 [PMID: 16491078 DOI: 10.1038/nn1657]
15 **Shen Q**, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 2004; **304**: 1338-1340 [PMID: 15060285 DOI: 10.1126/science.1095505]
16 **Silva-Vargas V**, Crouch EE, Doetsch F. Adult neural stem cells and their niche: a dynamic duo during homeostasis, regeneration, and aging. *Curr Opin Neurobiol* 2013; **23**: 935-942 [PMID: 24090877 DOI: 10.1016/j.conb.2013.09.004]
17 **Doetsch F**, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999; **97**: 703-716 [PMID: 10380923]
18 **Tavazoie M**, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 2008; **3**: 279-288 [PMID: 18786415 DOI: 10.1016/j.stem.2008.07.025]
19 **Mirzadeh Z**, Merkle FT, Soriano-Navarro M, Garcia-Verdugo JM, Alvarez-Buylla A. Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell* 2008; **3**: 265-278 [PMID: 18786414 DOI: 10.1016/j.stem.2008.07.004]
20 **Ihrie RA**, Alvarez-Buylla A. Lake-front property: a unique germinal niche by the lateral ventricles of the adult brain. *Neuron* 2011; **70**: 674-686 [PMID: 21609824 DOI: 10.1016/j.neuron.2011.05.004]
21 **Lois C**, Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. *Science* 1994; **264**: 1145-1148 [PMID: 8178174]
22 **Ming GL**, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 2011; **70**: 687-702 [PMID: 21609825 DOI: 10.1016/j.neuron.2011.05.001]
23 **Zhao C**, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. *Cell* 2008; **132**: 645-660 [PMID: 18295581 DOI: 10.1016/j.cell.2008.01.033]
24 **Cayre M**, Courtès S, Martineau F, Giordano M, Arnaud K, Zamaron A, Durbec P. Netrin 1 contributes to vascular remodeling in the subventricular zone and promotes progenitor emigration after demyelination. *Development* 2013; **140**: 3107-3117 [PMID: 23824572 DOI: 10.1242/dev.092999]
25 **Menn B**, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, Alvarez-Buylla A. Origin of oligodendrocytes in the subventricular zone of the adult brain. *J Neurosci* 2006; **26**: 7907-7918 [PMID: 16870736 DOI: 10.1523/JNEUROSCI.1299-06.2006]
26 **Bonaguidi MA**, Song J, Ming GL, Song H. A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. *Curr Opin Neurobiol* 2012; **22**: 754-761 [PMID: 22503352 DOI: 10.1016/j.conb.2012.03.013]
27 **Encinas JM**, Sierra A, Valcárcel-Martín R, Martín-Suárez S. A developmental perspective on adult hippocampal neurogenesis. *Int J Dev Neurosci* 2013; **31**: 640-645 [PMID: 23588197 DOI: 10.1016/j.ijdevneu.2013.04.001]
28 **Gage FH**. Mammalian neural stem cells. *Science* 2000; **287**: 1433-1438 [PMID: 10688783]
29 **Palmer TD**, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 2000; **425**: 479-494 [PMID: 10975875]
30 **Seri B**, García-Verdugo JM, McEwen BS, Alvarez-Buylla A. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* 2001; **21**: 7153-7160 [PMID: 11549726]
31 **Suh H**, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH. In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem Cell* 2007; **1**: 515-528 [PMID: 18371391 DOI: 10.1016/j.stem.2007.09.002]
32 **Bonaguidi MA**, Wheeler MA, Shapiro JS, Stadel RP, Sun GJ, Ming GL, Song H. In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell* 2011; **145**: 1142-1155 [PMID: 21664664 DOI: 10.1016/j.cell.2011.05.024]
33 **Encinas JM**, Michurina TV, Peunova N, Park JH, Tordo J, Peterson DA, Fishell G, Koulakov A, Enikolopov G. Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* 2011; **8**: 566-579 [PMID: 21549330 DOI: 10.1016/j.stem.2011.03.010]
34 **Bird A**. Perceptions of epigenetics. *Nature* 2007; **447**: 396-398 [PMID: 17522671 DOI: 10.1038/nature05913]
35 **Jaenisch R**, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003; **33** Suppl: 245-254 [PMID: 12610534 DOI: 10.1038/ng1089]
36 **Kouzarides T**. Chromatin modifications and their function. *Cell* 2007; **128**: 693-705 [PMID: 17320507 DOI: 10.1016/j.cell.2007.02.005]
37 **Strahl BD**, Allis CD. The language of covalent histone modifications. *Nature* 2000; **403**: 41-45 [PMID: 10638745 DOI: 10.1038/47412]
38 **Valk-Lingbeek ME**, Bruggeman SW, van Lohuizen M. Stem cells and cancer; the polycomb connection. *Cell* 2004; **118**: 409-418 [PMID: 15315754 DOI: 10.1016/j.cell.2004.08.005]
39 **Schilling E**, Rehli M. Global, comparative analysis of tissue-specific promoter CpG methylation. *Genomics* 2007; **90**: 314-323 [PMID: 17582736 DOI: 10.1016/j.ygeno.2007.04.011]
40 **Shen L**, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP. Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genet* 2007; **3**: 2023-2036 [PMID: 17967063 DOI: 10.1371/journal.pgen.0030181]
41 **Bird A**. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**: 6-21 [PMID: 11782440 DOI: 10.1101/gad.947102]
42 **Hendrich B**, Tweedie S. The methyl-CpG binding domain and the evolving role of DNA methylation in animals. *Trends Genet* 2003; **19**: 269-277 [PMID: 12711219 DOI: 10.1016/S0168-9525(03)00080-5]
43 **Tost J**. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Methods Mol Biol* 2009; **507**: 3-20 [PMID: 18987802 DOI: 10.1007/978-1-59745-522-0_1]
44 **Zhao X**, Ueba T, Christie BR, Barkho B, McConnell MJ, Nakashima K, Lein ES, Eadie BD, Willhoite AR, Muotri AR, Summers RG, Chun J, Lee KF, Gage FH. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. *Proc Natl Acad Sci USA* 2003; **100**: 6777-6782 [PMID: 12748381 DOI: 10.1073/pnas.1131928100]
45 **Bestor TH**. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J* 1992; **11**: 2611-2617 [PMID: 1628623]
46 **Pradhan S**, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem* 1999; **274**: 33002-33010 [PMID: 10551868]
47 **Brooks PJ**, Marietta C, Goldman D. DNA mismatch repair and DNA methylation in adult brain neurons. *J Neurosci* 1996; **16**: 939-945 [PMID: 8558262]
48 **Feng J**, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, Silva AJ, Fan G. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci* 2010; **13**:

423-430 [PMID: 20228804 DOI: 10.1038/nn.2514]
49 **Goto K**, Numata M, Komura JI, Ono T, Bestor TH, Kondo H. Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* 1994; **56**: 39-44 [PMID: 8026645]
50 **Hutnick LK**, Golshani P, Namihira M, Xue Z, Matynia A, Yang XW, Silva AJ, Schweizer FE, Fan G. DNA hypomethylation restricted to the murine forebrain induces cortical degeneration and impairs postnatal neuronal maturation. *Hum Mol Genet* 2009; **18**: 2875-2888 [PMID: 19433415 DOI: 10.1093/hmg/ddp222]
51 **Fan G**, Beard C, Chen RZ, Csankovszki G, Sun Y, Siniaia M, Biniszkiewicz D, Bates B, Lee PP, Kuhn R, Trumpp A, Poon C, Wilson CB, Jaenisch R. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. *J Neurosci* 2001; **21**: 788-797 [PMID: 11157065]
52 **Okano M**, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999; **99**: 247-257 [PMID: 10555141]
53 **Wu H**, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science* 2010; **329**: 444-448 [PMID: 20651149 DOI: 10.1126/science.1190485]
54 **Bai S**, Ghoshal K, Datta J, Majumder S, Yoon SO, Jacob ST. DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2. *Mol Cell Biol* 2005; **25**: 751-766 [PMID: 15632075 DOI: 10.1128/MCB.25.2.751-766.2005]
55 **Sikorska M**, Sandhu JK, Deb-Rinker P, Jezierski A, Leblanc J, Charlebois C, Ribecco-Lutkiewicz M, Bani-Yaghoub M, Walker PR. Epigenetic modifications of SOX2 enhancers, SRR1 and SRR2, correlate with in vitro neural differentiation. *J Neurosci Res* 2008; **86**: 1680-1693 [PMID: 18293417 DOI: 10.1002/jnr.21635]
56 **Ballas N**, Grunseich C, Lu DD, Speh JC, Mandel G. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* 2005; **121**: 645-657 [PMID: 15907476]
57 **Lunyak VV**, Burgess R, Prefontaine GG, Nelson C, Sze SH, Chenoweth J, Schwartz P, Pevzner PA, Glass C, Mandel G, Rosenfeld MG. Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* 2002; **298**: 1747-1752 [PMID: 12399542 DOI: 10.1126/science.1076469]
58 **Namihira M**, Nakashima K, Taga T. Developmental stage dependent regulation of DNA methylation and chromatin modification in a immature astrocyte specific gene promoter. *FEBS Lett* 2004; **572**: 184-188 [PMID: 15304345 DOI: 10.1016/j.febslet.2004.07.029]
59 **Sun Y**, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 2001; **104**: 365-376 [PMID: 11239394]
60 **Takizawa T**, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev Cell* 2001; **1**: 749-758 [PMID: 11740937]
61 **Fan G**, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H, ten Hoeve J, Shuai K, Sun YE. DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. *Development* 2005; **132**: 3345-3356 [PMID: 16014513 DOI: 10.1242/dev.01912]
62 **Seisenberger S**, Peat JR, Hore TA, Santos F, Dean W, Reik W. Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. *Philos Trans R Soc Lond B Biol Sci* 2013; **368**: 20110330 [PMID: 23166394 DOI: 10.1098/rstb.2011.0330]
63 **Ito T**. Role of histone modification in chromatin dynamics. *J Biochem* 2007; **141**: 609-614 [PMID: 17405795 DOI: 10.1093/jb/mvm091]
64 **Tahiliani M**, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009; **324**: 930-935 [PMID: 19372391 DOI: 10.1126/science.1170116]
65 **Cortellino S**, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A. Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. *Cell* 2011; **146**: 67-79 [PMID: 21722948 DOI: 10.1016/j.cell.2011.06.020]
66 **He YF**, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 2011; **333**: 1303-1307 [PMID: 21817016 DOI: 10.1126/science.1210944]
67 **Loenarz C**, Schofield CJ. Oxygenase catalyzed 5-methylcytosine hydroxylation. *Chem Biol* 2009; **16**: 580-583 [PMID: 19549596 DOI: 10.1016/j.chembiol.2009.06.002]
68 **Iqbal K**, Jin SG, Pfeifer GP, Szabó PE. Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proc Natl Acad Sci USA* 2011; **108**: 3642-3647 [PMID: 21321204 DOI: 10.1073/pnas.1014033108]
69 **Ito S**, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 2010; **466**: 1129-1133 [PMID: 20639862 DOI: 10.1038/nature09303]
70 **Mellén M**, Ayata P, Dewell S, Kriaucionis S, Heintz N. MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. *Cell* 2012; **151**: 1417-1430 [PMID: 23260135 DOI: 10.1016/j.cell.2012.11.022]
71 **Koh KP**, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A. Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell* 2011; **8**: 200-213 [PMID: 21295276 DOI: 10.1016/j.stem.2011.01.008]
72 **Kaas GA**, Zhong C, Eason DE, Ross DL, Vachhani RV, Ming GL, King JR, Song H, Sweatt JD. TET1 controls CNS 5-methylcytosine hydroxylation, active DNA demethylation, gene transcription, and memory formation. *Neuron* 2013; **79**: 1086-1093 [PMID: 24050399 DOI: 10.1016/j.neuron.2013.08.032]
73 **Rudenko A**, Dawlaty MM, Seo J, Cheng AW, Meng J, Le T, Faull KF, Jaenisch R, Tsai LH. Tet1 is critical for neuronal activity-regulated gene expression and memory extinction. *Neuron* 2013; **79**: 1109-1122 [PMID: 24050401 DOI: 10.1016/j.neuron.2013.08.003]
74 **Zhang RR**, Cui QY, Murai K, Lim YC, Smith ZD, Jin S, Ye P, Rosa L, Lee YK, Wu HP, Liu W, Xu ZM, Yang L, Ding YQ, Tang F, Meissner A, Ding C, Shi Y, Xu GL. Tet1 regulates adult hippocampal neurogenesis and cognition. *Cell Stem Cell* 2013; **13**: 237-245 [PMID: 23770080 DOI: 10.1016/j.stem.2013.05.006]
75 **Luger K**, Richmond TJ. DNA binding within the nucleosome core. *Curr Opin Struct Biol* 1998; **8**: 33-40 [PMID: 9519294]
76 **Olins DE**, Olins AL. Chromatin history: our view from the bridge. *Nat Rev Mol Cell Biol* 2003; **4**: 809-814 [PMID: 14570061 DOI: 10.1038/nrm1225]
77 **Tropberger P**, Schneider R. Scratching the (lateral) surface of chromatin regulation by histone modifications. *Nat Struct Mol Biol* 2013; **20**: 657-661 [PMID: 23739170 DOI: 10.1038/nsmb.2581]
78 **Marmorstein R**, Trievel RC. Histone modifying enzymes: structures, mechanisms, and specificities. *Biochim Biophys Acta* 2009; **1789**: 58-68 [PMID: 18722564 DOI: 10.1016/j.bbagrm.2008.07.009]
79 **Bannister AJ**, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011; **21**: 381-395 [PMID: 21321607 DOI: 10.1038/cr.2011.22]
80 **Foti SB**, Chou A, Moll AD, Roskams AJ. HDAC inhibitors dysregulate neural stem cell activity in the postnatal mouse brain. *Int J Dev Neurosci* 2013; **31**: 434-447 [PMID: 23542004 DOI: 10.1016/j.ijdevneu.2013.03.008]

81 **Jawerka M**, Colak D, Dimou L, Spiller C, Lagger S, Montgomery RL, Olson EN, Wurst W, Göttlicher M, Götz M. The specific role of histone deacetylase 2 in adult neurogenesis. *Neuron Glia Biol* 2010; **6**: 93-107 [PMID: 20388229 DOI: 10.1017/S1740925X10000049]

82 **Siebzehnrubl FA**, Buslei R, Eyupoglu IY, Seufert S, Hahnen E, Blumcke I. Histone deacetylase inhibitors increase neuronal differentiation in adult forebrain precursor cells. *Exp Brain Res* 2007; **176**: 672-678 [PMID: 17216146 DOI: 10.1007/s00221-006-0831-x]

83 **He Y**, Sandoval J, Casaccia-Bonnefil P. Events at the transition between cell cycle exit and oligodendrocyte progenitor differentiation: the role of HDAC and YY1. *Neuron Glia Biol* 2007; **3**: 221-231 [PMID: 18634613 DOI: 10.1017/S1740925X08000057]

84 **Lyssiotis CA**, Walker J, Wu C, Kondo T, Schultz PG, Wu X. Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. *Proc Natl Acad Sci U S A* 2007; **104**: 14982-14987 [PMID: 17855562 DOI: 10.1073/pnas.0707044104]

85 **Li B**, Carey M, Workman JL. The role of chromatin during transcription. *Cell* 2007; **128**: 707-719 [PMID: 17320508 DOI: 10.1016/j.cell.2007.01.015]

86 **Tsukada Y**, Ishitani T, Nakayama KI. KDM7 is a dual demethylase for histone H3 Lys 9 and Lys 27 and functions in brain development. *Genes Dev* 2010; **24**: 432-437 [PMID: 20194436 DOI: 10.1101/gad.1864410]

87 **Zhang J**, Ji F, Liu Y, Lei X, Li H, Ji G, Yuan Z, Jiao J. Ezh2 regulates adult hippocampal neurogenesis and memory. *J Neurosci* 2014; **34**: 5184-5199 [PMID: 24719098 DOI: 10.1523/JNEUROSCI.4129-13.2014]

88 **Park DH**, Hong SJ, Salinas RD, Liu SJ, Sun SW, Sgualdino J, Testa G, Matzuk MM, Iwamori N, Lim DA. Activation of neuronal gene expression by the JMJD3 demethylase is required for postnatal and adult brain neurogenesis. *Cell Rep* 2014; **8**: 1290-1299 [PMID: 25176653 DOI: 10.1016/j.celrep.2014.07.060]

89 **Foret MR**, Sandstrom RS, Rhodes CT, Wang Y, Berger MS, Lin CH. Molecular targets of chromatin repressive mark H3K9me3 in primate progenitor cells within adult neurogenic niches. *Front Genet* 2014; **5**: 252 [PMID: 25126093 DOI: 10.3389/fgene.2014.00252]

90 **Lim DA**, Huang YC, Swigut T, Mirick AL, Garcia-Verdugo JM, Wysocka J, Ernst P, Alvarez-Buylla A. Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature* 2009; **458**: 529-533 [PMID: 19212323 DOI: 10.1038/nature07726]

91 **Gonzales-Roybal G**, Lim DA. Chromatin-based epigenetics of adult subventricular zone neural stem cells. *Front Genet* 2013; **4**: 194 [PMID: 24115953 DOI: 10.3389/fgene.2013.00194]

92 **Ferguson-Smith AC**. Genomic imprinting: the emergence of an epigenetic paradigm. *Nat Rev Genet* 2011; **12**: 565-575 [PMID: 21765458 DOI: 10.1038/nrg3032]

93 **Edwards CA**, Ferguson-Smith AC. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007; **19**: 281-289 [PMID: 17467259 DOI: 10.1016/j.ceb.2007.04.013]

94 **McEwen KR**, Ferguson-Smith AC. Distinguishing epigenetic marks of developmental and imprinting regulation. *Epigenetics Chromatin* 2010; **3**: 2 [PMID: 20180964 DOI: 10.1186/1756-8935-3-2]

95 **Hirasawa R**, Feil R. Genomic imprinting and human disease. *Essays Biochem* 2010; **48**: 187-200 [PMID: 20822494 DOI: 10.1042/bse0480187]

96 **Ferrón SR**, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, Morante-Redolat JM, Laborda J, Guillemot F, Bauer SR, Fariñas I, Ferguson-Smith AC. Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. *Nature* 2011; **475**: 381-385 [PMID: 21776083 DOI: 10.1038/nature10229]

97 **DeChiara TM**, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991; **64**: 849-859 [PMID: 1997210]

98 **Giannoukakis N**, Deal C, Paquette J, Goodyer CG, Polychronakos C. Parental genomic imprinting of the human IGF2 gene. *Nat Genet* 1993; **4**: 98-101 [PMID: 8099843 DOI: 10.1038/ng0593-98]

99 **Lehtinen MK**, Zappaterra MW, Chen X, Yang YJ, Hill AD, Lun M, Maynard T, Gonzalez D, Kim S, Ye P, D'Ercole AJ, Wong ET, LaMantia AS, Walsh CA. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron* 2011; **69**: 893-905 [PMID: 21382550 DOI: 10.1016/j.neuron.2011.01.023]

100 **Krishnakumar R**, Blelloch RH. Epigenetics of cellular reprogramming. *Curr Opin Genet Dev* 2013; **23**: 548-555 [PMID: 23948105 DOI: 10.1016/j.gde.2013.06.005]

101 **Mikkelsen TS**, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A. Dissecting direct reprogramming through integrative genomic analysis. *Nature* 2008; **454**: 49-55 [PMID: 18509334 DOI: 10.1038/nature07056]

102 **Chin MH**, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell* 2009; **5**: 111-123 [PMID: 19570518 DOI: 10.1016/j.stem.2009.06.008]

103 **Papp B**, Plath K. Epigenetics of reprogramming to induced pluripotency. *Cell* 2013; **152**: 1324-1343 [PMID: 23498940 DOI: 10.1016/j.cell.2013.02.043]

104 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]

105 **Hester ME**, Song S, Miranda CJ, Eagle A, Schwartz PH, Kaspar BK. Two factor reprogramming of human neural stem cells into pluripotency. *PLoS One* 2009; **4**: e7044 [PMID: 19763260 DOI: 10.1371/journal.pone.0007044]

106 **Maherali N**, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 2007; **1**: 55-70 [PMID: 18371336 DOI: 10.1016/j.stem.2007.05.014]

107 **Park IH**, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008; **451**: 141-146 [PMID: 18157115 DOI: 10.1038/nature06534]

108 **Wernig M**, Meissner A, Cassady JP, Jaenisch R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2008; **2**: 10-12 [PMID: 18371415 DOI: 10.1016/j.stem.2007.12.001]

109 **Aoi T**, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 2008; **321**: 699-702 [PMID: 18276851 DOI: 10.1126/science.1154884]

110 **Hanna J**, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creyghton MP, Steine EJ, Cassady JP, Foreman R, Lengner CJ, Dausman JA, Jaenisch R. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* 2008; **133**: 250-264 [PMID: 18423197 DOI: 10.1016/j.cell.2008.03.028]

111 **Stadtfeld M**, Brennand K, Hochedlinger K. Reprogramming of pancreatic beta cells into induced pluripotent stem cells. *Curr Biol* 2008; **18**: 890-894 [PMID: 18501604 DOI: 10.1016/j.cub.2008.05.010]

112 **Eminli S**, Utikal J, Arnold K, Jaenisch R, Hochedlinger K. Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. *Stem Cells* 2008; **26**: 2467-2474 [PMID: 18635867 DOI: 10.1634/stemcells.2008-0317]

113 **Kim JB**, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, Ehrich M, van den Boom D, Meyer J, Hübner K, Bernemann C, Ortmeier C, Zenke M, Fleischmann BK, Zaehres H, Schöler HR. Oct4-induced pluripotency in adult neural stem cells. *Cell* 2009; **136**: 411-419 [PMID: 19203577 DOI: 10.1016/j.cell.2009.01.023]

114 **Kim JB**, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, Araúzo-Bravo MJ, Ruau D, Han DW, Zenke M, Schöler HR. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 2008; **454**: 646-650 [PMID: 18594515 DOI: 10.1038/nature07061]

115 **Lin SL**, Chang DC, Lin CH, Ying SY, Leu D, Wu DT. Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Res* 2011; **39**: 1054-1065 [PMID: 20870751 DOI: 10.1093/nar/gkq850]
116 **Lee HJ**, Hore TA, Reik W. Reprogramming the methylome: erasing memory and creating diversity. *Cell Stem Cell* 2014; **14**: 710-719 [PMID: 24905162 DOI: 10.1016/j.stem.2014.05.008]
117 **Hochedlinger K**, Plath K. Epigenetic reprogramming and induced pluripotency. *Development* 2009; **136**: 509-523 [PMID: 19168672 DOI: 10.1242/dev.020867]
118 **Singh RP**, Shiue K, Schomberg D, Zhou FC. Cellular epigenetic modifications of neural stem cell differentiation. *Cell Transplant* 2009; **18**: 1197-1211 [PMID: 19660178 DOI: 10.3727/096368909X12483162197204]
119 **Shi Y**, Desponts C, Do JT, Hahm HS, Schöler HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell* 2008; **3**: 568-574 [PMID: 18983970 DOI: 10.1016/j.stem.2008.10.004]
120 **Apostolou E**, Hochedlinger K. Chromatin dynamics during cellular reprogramming. *Nature* 2013; **502**: 462-471 [PMID: 24153299 DOI: 10.1038/nature12749]
121 **Theunissen TW**, Jaenisch R. Molecular control of induced pluripotency. *Cell Stem Cell* 2014; **14**: 720-734 [PMID: 24905163 DOI: 10.1016/j.stem.2014.05.002]
122 **Wernig M**, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007; **448**: 318-324 [PMID: 17554336 DOI: 10.1038/nature05944]
123 **Kim K**, Zhao R, Doi A, Ng K, Unternaehrer J, Cahan P, Huo H, Loh YH, Aryee MJ, Lensch MW, Li H, Collins JJ, Feinberg AP, Daley GQ. Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nat Biotechnol* 2011; **29**: 1117-1119 [PMID: 22119740 DOI: 10.1038/nbt.2052]
124 **Tobin SC**, Kim K. Generating pluripotent stem cells: differential epigenetic changes during cellular reprogramming. *FEBS Lett* 2012; **586**: 2874-2881 [PMID: 22819821 DOI: 10.1016/j.febslet.2012.07.024]
125 **Ying QL**, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature* 2008; **453**: 519-523 [PMID: 18497825 DOI: 10.1038/nature06968]
126 **Ficz G**, Hore TA, Santos F, Lee HJ, Dean W, Arand J, Krueger F, Oxley D, Paul YL, Walter J, Cook SJ, Andrews S, Branco MR, Reik W. FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency. *Cell Stem Cell* 2013; **13**: 351-359 [PMID: 23850245 DOI: 10.1016/j.stem.2013.06.004]
127 **Habibi E**, Brinkman AB, Arand J, Kroeze LI, Kerstens HH, Matarese F, Lepikhov K, Gut M, Brun-Heath I, Hubner NC, Benedetti R, Altucci L, Jansen JH, Walter J, Gut IG, Marks H, Stunnenberg HG. Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* 2013; **13**: 360-369 [PMID: 23850244 DOI: 10.1016/j.stem.2013.06.002]
128 **Leitch HG**, Tang WW, Surani MA. Primordial germ-cell development and epigenetic reprogramming in mammals. *Curr Top Dev Biol* 2013; **104**: 149-187 [PMID: 23587241 DOI: 10.1016/B978-0-12-416027-9.00005-X]

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REVIEW

# Epidermal stem cells and skin tissue engineering in hair follicle regeneration

María Eugenia Balañá, Hernán Eduardo Charreau, Gustavo José Leirós

María Eugenia Balañá, Gustavo José Leirós, Fundación Pablo Cassará -Instituto de Ciencia y Tecnología Dr. César Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina
Hernán Eduardo Charreau, Technology Intelligence Unit, Clarke, Modet & C°, Lavalle 190, 3° Piso, C1047AAD Buenos Aires, Argentina
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Correspondence to: Dr. María Eugenia Balañá, Fundación Pablo Cassará, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina. mbalana@fundacioncassara.org.ar
Telephone: +54-11-41054127
Fax: +54-11-46874065
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## Abstract

The reconstitution of a fully organized and functional hair follicle from dissociated cells propagated under defined tissue culture conditions is a challenge still pending in tissue engineering. The loss of hair follicles caused by injuries or pathologies such as alopecia not only affects the patients' psychological well-being, but also endangers certain inherent functions of the skin. It is then of great interest to find different strategies aiming to regenerate or neogenerate the hair follicle under conditions proper of an adult individual. Based upon current knowledge on the epithelial and dermal cells and their interactions during the embryonic hair generation and adult hair cycling, many researchers have tried to obtain mature hair follicles using different strategies and approaches depending on the causes of hair loss. This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, with emphasis on those involving neogenesis of hair follicles in adult individuals using isolated cells and tissue engineering. Most of these experiments were performed using rodent cells, particularly from embryonic or newborn origin. However, no successful strategy to generate human hair follicles from adult cells has yet been reported. This review identifies several issues that should be considered to achieve this objective. Perhaps the most important challenge is to provide three-dimensional culture conditions mimicking the structure of living tissue. Improving culture conditions that allow the expansion of specific cells while protecting their inductive properties, as well as methods for selecting populations of epithelial stem cells, should give us the necessary tools to overcome the difficulties that constrain human hair follicle neogenesis. An analysis of patent trends shows that the number of patent applications aimed at hair follicle regeneration and neogenesis has been increasing during the last decade. This field is attractive not only to academic researchers but also to the companies that own almost half of the patents in this field.

**Key words:** Adult stem cells; Skin grafts; Epidermis; Multipotential differentiation; Tissue regeneration; Dermal papilla; Epithelial-mesenchymal interactions

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**Core tip:** Loss of hair follicles caused by injuries or pathologies affects the patients' psychological well-being and endangers inherent functions of the skin. Different experimental strategies and approaches to obtain mature hair follicles have been designed based upon current knowledge of the epithelial and dermal cells involved in embryonic hair generation and adult hair cycling, and in the epithelial-mesenchymal interactions among them. This review summarizes the current advances in hair follicle neogenesis and regeneration, with emphasis on those involving neogenesis of hair follicles in adults from isolated cells and tissue engineering as well as an analysis on patent trends in this field.

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## INTRODUCTION

Regenerative medicine aims to create living, functional tissues that repair or replace lost or damaged organ function resulting from disease, injury, congenital defects or aging.

The main challenge of tissue engineering is the reconstitution of fully organized and functional organ systems from dissociated cells that have been propagated under defined tissue culture conditions.

Skin is the largest organ in the human body, acting as a barrier with protective, immunologic and sensorial functions. Deep skin injuries produce a complete destruction of skin regenerative elements. These wounds heal by contraction, with epithelization from the edges only and extensive scarring, resulting in reduced joint movements and cosmetic defects[1]. Moreover, if these lesions are too extensive, the healing process is unsuccessful and they become life-threatening for the patient. Tissue engineering has emerged as a new interdisciplinary field combining scaffolds, cells and biomolecular signals towards the treatment of skin lesions. This useful strategy may contribute not only to the treatment of deep skin injuries but also to the understanding of skin regeneration.

The main goal of tissue-engineered skin grafts is to restore lost barrier function. Many dermal-epidermal composites or skin equivalents have been described for use in the clinic[2] but the inability of these skin constructs to regenerate skin appendages as hair follicles has limited their use. Although the ability to reconstitute adult skin with functional skin appendages has long been a major clinical objective, the regeneration of epidermal appendages, such as hair follicles, and sebaceous and sweat glands, is a challenge that is still pending.

Recent studies clearly demonstrate that there are multipotent stem cells with the capability to regenerate hair follicles and sebaceous glands in adult mammalian skin and this multipotency can be maintained in cell culture[3,4]. The hair-differentiation potentiality of epidermal stem cells can be activated by inductive dermal cells. As will be extensively reviewed in this article, co-grafting of those cellular components from mice allows complete hair reconstitution[5]. The successful regeneration of hair follicles in immunodeficient mice suggests that creating human hair follicles in tissue-engineered skin grafts is feasible. However, regeneration of human hair in a similar manner has not been reported.

Skin appendages cannot be restored in healed wounds by current tissue-engineered skin grafts[6] mainly because of their limited self-regeneration capability in adults[7], the lack of appendage structures in the human skin grafts and probably an improper molecular microenvironment. Many strategies still need to be explored, particularly enriching isolated cells with trichogenic capability, maintaining this capability during processing, and providing the cells with proper environmental signals.

The lack of skin appendages by skin wounds and by pathologies such as different kinds of alopecia not only affects the patients' psychological well-being[8], but also endangers the inherent functions of the skin.

Skin appendages cannot regenerate on their own after damage[7]. For the fully functional regeneration of ectodermal organs, it has been proposed that a bioengineered organ could be developed by reproducing the embryonic processes of organogenesis.

This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, *in vitro* and *in vivo*, with emphasis on approaches that include the neogenesis of hair follicles in adults from isolated cells and by tissue engineering as well as an analysis on patent trends in this field.

## ANATOMY AND BIOLOGY OF HAIR FOLLICLES

The skin consists of three layers: epidermis, dermis, and hypodermis. The epidermis is in fact a multi-layered epithelium extending from the basement membrane that separates it from the dermis to the air. The dermis is located below the epidermis and is a connective tissue comprised of extracellular matrix, fibroblasts, vascular endothelial cells, and skin appendages.

Skin appendages, including hair follicles, sebaceous glands and sweat glands, are linked to the epidermis but project deep into the dermal layer. A human has around five million hair follicles with two types of hair, terminal hair (long, thick pigmented) and vellus hair (thin, unpigmented)[9].

Hair follicles are composed of an outer root sheath

(ORS) that is contiguous with the epidermis, an inner root sheath (IRS) and the hair shaft (HS). The matrix surrounding the dermal papilla (DP), in the hair root, contains actively dividing and relatively undifferentiated cells that are essential for follicle formation.

In embryogenesis, the skin begins as a single layer of epidermal stem cells. Then, mesenchymal cells populate the skin to form the underlying collagenous dermis[10]. Human hair follicles start to develop through complex morphogenetic processes resulting from highly coordinated series of bidirectional epithelial-mesenchymal interactions[11]. Hair follicle development is initiated by the appearance of a thickening in the embryonic ectoderm called placode resulting from the condensation of the underlying mesoderm that will form the DP[12]. The DP becomes a permanent part of the follicle base[13] enveloped by the hair bulb. It is considered the commander of the hair follicle determining the hair thickness, length, and life cycle[8]. Signals from the condensed mesoderm induce the proliferation of the placode which forms mature hair follicles by a systematic series of differentiation and proliferation processes of epithelial cells. The hair follicle becomes fully mature when its bulb nears the bottom of the dermis. At this point, the proliferative cells (matrix) at the follicle base continue to divide, producing progeny cells that terminally differentiate to form the growing hair that emerges from the skin surface. The inner layers begin to differentiate into concentric cylinders to form the central HS and the surrounding channel, the IRS.

These tissues undergo continual rejuvenation and, in response to injury, they must be prepared for wound repair. The capability of the skin for maintaining tissue homeostasis, regenerating hair, and repairing the epidermis after injury resides in its stem cells.

## HAIR FOLLICLE STEM CELLS AND THE HAIR CYCLE

Stem cells are undifferentiated cells that are ultimately responsible for the constant renewal of the skin due to their distinguishing properties of self-renewal for the entire life span of an organism and their ability to differentiate into a variety of specialized cells.

A pool of progenitor cells is located on the basement membrane of the skin. These cells contribute to epidermal homeostasis undergoing continuous self-renewal and differentiation to keratinocytes that migrate towards the surface of the skin where they undergo terminal differentiation and maturation providing the skin's barrier properties.

Another reservoir of slow-cycling multipotent stem cells that gives rise to a range of differentiated cell types in skin is located in a specialized region of the ORS in the hair follicle, known as the bulge[3,4,14-16]. Early work showed that these slow cycling multipotent stem cells not only contribute to the growth of hair follicles themselves and of the sebaceous glands[3] but also can be activated and migrate out of hair follicles in order to repair the damaged epithelium[17]; however, they contribute little to the turnover of the intact epidermis. In the absence of these hair follicle stem cells (HFSC), hair follicle and sebaceous gland morphogenesis is blocked, and epidermal wound repair is compromised[18]. The epithelial-mesenchymal interactions with the underlying DP play a pivotal role in embryonic hair genesis[12], in the regulation of post-natal hair follicle cyclical activity, and in the repair of wounded skin[19-22].

Unlike most organs, hair follicles do not reach homeostasis once they mature. Each mature hair follicle is a regenerating system, which physiologically undergoes cycles of growth (anagen), regression (catagen), and rest (telogen) numerous times in adult life[23]. In catagen, hair follicle stem cells are maintained in the bulge. Then, the resting follicle re-enters anagen (regeneration) when proper molecular signals are provided. During late telogen to early anagen transition, signals from the DP stimulate the hair germ and quiescent bulge stem cells to become activated[24]. Many paracrine factors are involved in this crosstalk at different hair cycle stages and some signaling pathways have been implicated[3,19,25]. In anagen, stem cells in the bulge give rise to hair germs, then the transient amplifying cells in the matrix of the new follicle proliferate rapidly to form a new hair filament[26].

Even if it is generally believed that hair follicles do not form after birth in humans[27], the formation of vellus hair follicles from the reconstituted epidermis of an abraded area of facial skin was described in 1956[28]. However, the great challenge of skin tissue engineering and skin wound regeneration remains to be its inability to reliably reconstitute skin appendages, most notably hair follicles and sweat glands. The information about the mechanisms that generate and maintain skin appendages provided by recent studies with skin-derived progenitor cells, may be the basis of new therapies that could help to overcome these limitations.

## CAUSES OF HAIR LOSS AND REGENERATION OF THE HAIR FOLLICLE

Hair loss responds to different causes ranging from mild traumas, such as hair fiber plucking, to severe traumas such as partial- or full-thickness skin lesions. Pathological processes, such as cicatricial or non cicatricial alopecia, can also be responsible for hair loss. After plucking, if HFSC and DP remain, a new hair filament would be spontaneously regenerated. Some label retaining studies have shown that hair follicle stem cells in the bulge remain intact after hair plucking[29,30] whereas others reported bulge stem cells suffering apoptosis after plucking which are replaced by label retaining cells coming from hair germ[31].

It was also observed that the slow-cycling bulge cells (CD34 positive cells in mouse) are involved in normal hair homeostasis and wound healing, whereas regeneration after hair plucking involved actively cycling cells from the lower ORS[26]. However, the cell dynamics in this process is less clear than in the physiological renewal and further studies are required to understand this process.

However, when the cellular niches are completely lost, it is necessary to generate a completely new hair follicle in a process called hair follicle neogenesis.

Based on the knowledge on the epithelial and dermal cells, and their interactions, during the embryonic hair generation and adult hair cycling, different experimental approaches have been designed to regenerate hair follicles or generate new ones by the neogenesis process.

These hair regeneration and neogenesis attempts are recapitulated in this article, and can be classified into 4 categories: (1) reversion of pathological intra- and/or extra-follicular environment, for instance androgenetic alopecia (AGA); (2) regeneration of complete hair follicles from the recombination of hair follicle parts; (3) neogenesis of hair follicles from isolated cells; and (4) neogenesis of hair follicles by tissue engineering.

### *Reversion of pathological intra- and/or extra-follicular environment*

Hair follicle cycle and growth can be affected and deregulated by paracrine factors from the follicle itself and/or from the surrounding dermal tissue, or by endocrine factors[32,33] leading to hair loss. Many factors have been described as molecular mediators of hair follicle growth [11]. Some of them, such as PDGF[33] and Wnt proteins[34], are positive regulators while others, such as BMPs[35], suppress hair growth. Many paracrine factors involved in the epithelial-mesenchymal crosstalk at different stages of the hair cycle, and some implicated signaling pathways, have also been studied[3,19,25].

In addition, endocrine factors, such as sexual hormones, have both physiological and pathological effects on hair growth as in human male beard, and androgenetic alopecia respectively. Androgenetic alopecia[36] is a common, progressive disorder in which large, terminal scalp hairs are gradually replaced by smaller hairs following a defined pattern. The DP is the site through which androgens act on follicle cells by altering the regulatory paracrine factors involved in the differentiation and proliferation of HF stem cells. DPs from balding scalp have higher levels of androgen receptor and 5-alpha reductase. Dihydrotestosterone can induce DP to secrete factors including, TGF-beta1 and DKK-1 which down regulate keratinocytes growth[37,38].

A functional crosstalk between the androgen receptor and Wnt signaling pathways in DPCs has been described in target tissues[39]. Moreover, our group has demonstrated that androgens regulate secreted factors involved in normal HF stem cell differentiation via the inhibition of the canonical Wnt signaling system in androgen sensitive DPCs. We provided evidence that androgen activation of GSK-3β would be responsible for the inhibition of Wnt/β-catenin signaling[40]. The current pharmacological treatment for AGA includes androgen metabolism modulators, such as the 5-alpha reductase inhibitor finasteride and the antiandrogen agent flutamide. Nevertheless, the results of these treatments are not only variable and patient-dependent, but can also cause undesirable side effects. Therefore, therapies acting on specific molecular targets are necessary to treat this pathology downstream from the actual antiandrogenetic pharmaceutical modulators. The crosstalk between the androgen receptor (AR) and Wnt/β-catenin signaling pathways could be one alternative therapeutic target, among many others recently reviewed[41].

In these experimental approaches, modulation of the intra-follicular microenvironment and the extra-follicular macroenvironment could contribute to promote growth of the existing hair follicles but not to increase their number by the formation of new ones.

### *Regeneration of hair follicles by recombination of hair-follicle parts*

Taking into account the inductive ability of DP and the current knowledge on epithelial-mesenchymal interactions, different recombination of dermal and epidermal tissue have been employed for hair follicle regeneration. In the early 1960s, researchers showed that whole follicle end bulbs and isolated whole whisker papillae (containing dermal and epidermal components) remain viable and may produce whisker hair follicles in a new site[42]. The most surprising finding was that isolated DPs (without epidermal components) were capable of inducing the generation of new hair follicles recruiting epithelial cells from the implantation sites. The same results were obtained when epidermis from different origins were transplanted together with whisker DP[43]. Regeneration of hair follicles was also observed in both mice[43-45] and humans[46] when dermal sheath tissue was used, which was sufficient to regenerate also the DP structure. After implantation, the whisker DP was capable of inducing hair follicle regeneration retaining the information to determine hair fiber type and follicle size[47].

### *Neogenesis of hair follicles in adult individuals from isolated cells*

Grafting of dermal-inductive tissue is limited by the fact that it is not possible to generate more hair follicles than the obtained from the donor tissues. To overcome this limitation different approaches and experimental models using freshly or cultured isolated cells from both dermal and dermal/epidermal origin were tested. Most of them involved neonatal and embryonic murine cells. One of the first models used was the chamber assay (Figure 1) in which dermal and epithelial cells are seeded inside a chamber consisting of a cylinder

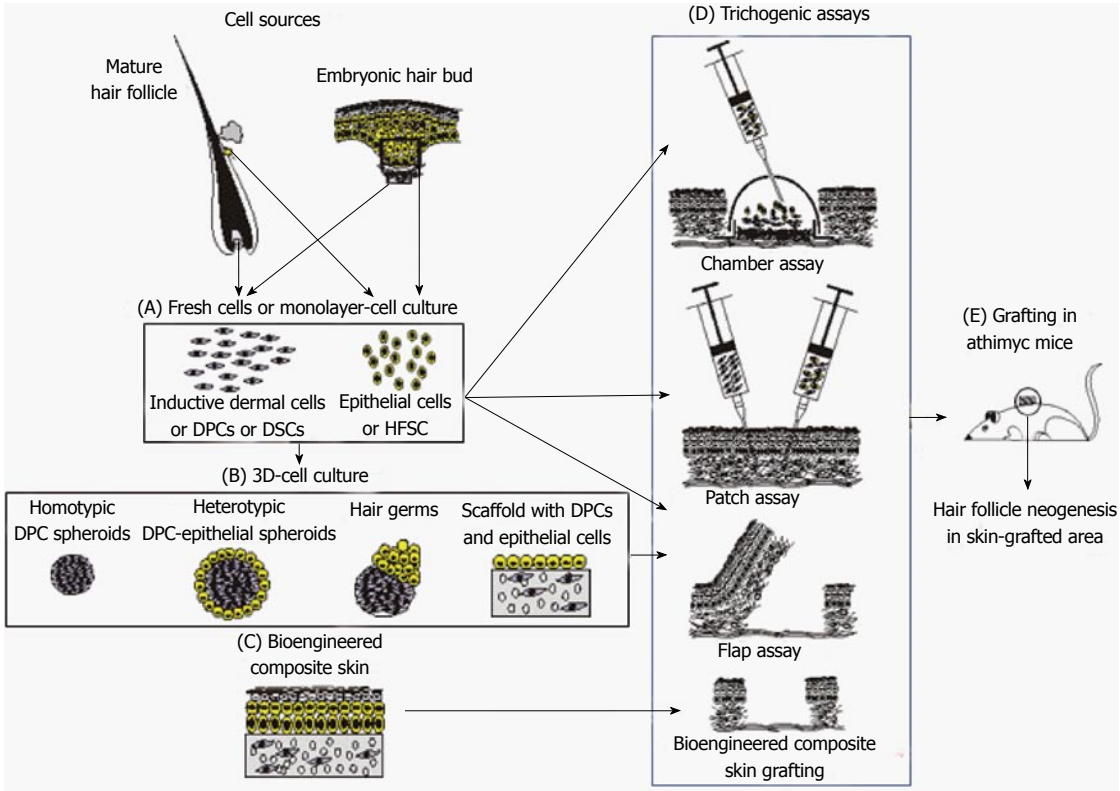


**Figure 1 Hair follicle neogenesis using isolated epithelial and dermal cells.** Inductive-dermal and Epithelial Cells can be obtained from adult tissue or embryonic sources. Isolated cells by enzymatic digestion of living tissues, or monolayer cell cultures (A) can be used in the trichogenic assays (D): Chamber assay; Patch assay; and flap assay. Moreover, isolated cells can be cultured in 3D-conditions, allowing them to create their own extracellular matrix as in the case of homotypic and heterotypic spheroids and hair germs (B). Also, cells can be seeded on a precast scaffold used for cellular support or used to produce a bioengineered composite skin (C). Any of these constructs can be evaluated by the trichogenic assays (D): Flap assay; and Bioengineered composite skin grafting. All these trichogenic assays are mainly performed in the back-skin of athymic mice in order to avoid immune rejections and to take advantage of its bare-skin (E). DPCs: Dermal papilla cells; DSCs: Dermal sheath cells; HFSC: Hair follicle stem cells.

inserted through a full-thickness skin lesion in mouse dorsal skin, covered by a dome. In former studies using this device in nude mice[48], it was showed that hair buds obtained from neonatal mice combined with fresh neonatal dermal cells or with immortalized clones from vibrissae rat DPCs produced mature and cycling hair follicles. Using the same chamber assay, hair bud preparations without any additional inductive dermal component were unable to form hair follicles resulting in a scarring reparative tissue, a thin epidermis and a dermis without appendages[49]. On the other hand, the combination of hair buds with fresh dermal cell preparations or early passages of cloned vibrissae cell lines resulted in skin with normal epidermal and dermal layers.

Although these studies confirmed the trichogenic potential of dissociated dermal cells, particularly DPCs, the conditions of the chamber assay correspond to a wound healing environment and do not represent the physiological status. Other approaches allow studying the trichogenic potential of isolated cells in a more physiological environment, as is the case of the patch assay (Figure 1). This assay consists in injecting dissociated murine neonatal epidermal and dermal cells hypodermically into adult mouse skin. This system, originally described by Zheng *et al*[50], showed the formation of cell clusters that resulted in "infundibular cysts" which generated hair germs, followed by pegs that grew to differentiate into cycling mature pilosebaceous units. Surprisingly, whereas patch hair follicles were in anagen phase, the host skin hair follicles were in telogen and vice versa, suggesting that follicle cycling depends on the trichogenic cells and not on the host skin hair cycling. Even when the shaft grows inside an intradermal cyst that tends to extinction by inflammatory processes, none of the other already described systems at that moment showed to be so efficient to evaluate the trichogenic potential of isolated cells. A variation of this method is called tracheal grafting method. It consists in inoculating rat tracheas with fresh or cultured keratinocytes from newborn mice together with newborn fibroblasts or cultured DPCs and implanting them subcutaneously in athymic mice for four weeks. In this assay hair follicles and sebaceous glands were generated using cultured keratinocytes from newborn mice and fresh newborn fibroblasts[51]. Although adult mice keratinocytes successfully produced a two layered epithelium, they failed to generate hair follicles or sebaceous glands[51].

Other strategy to evaluate the hair inductive capability of dermal cells is the "flap assay" (Figure 1). This

approach originally described by Qiao *et al*[52] consists in: (1) generating a skin flap in the dorsal skin of nude mice; (2) locating an embryonic epidermis supported by a silicone sheet in direct contact with the muscle with the basal side of the embryonic epidermis facing upwards; (3) seeding embryonic murine dermal cells or cultured DPCs onto the basal face of the graft-epidermis; and (4) pulling over the graft and suturing the flap edges. In this configuration the graft-dermal cells were layered between the graft-epidermis and the host connective tissue. When fresh embryonic dermal cells were used, abundant hair was developed four weeks after grafting. The induced hairs were oriented vertically down towards the silicone sheet. After surgical flap inversion, the formed hairs were exposed in the host skin surface and survived for more than 12 mo.

### *Neogenesis of hair follicles by tissue engineering*

Although the assays described above provided much information respect to the hair neogenesis process and the cells involved in it, the high number of cells required, the need of *in vivo* animal models and the poor results obtained with human cellular sources make these procedures not applicable for clinical uses in the near future. The development of new strategies has become necessary and begun to emerge. They are mainly focused on tissue engineering-based follicle neogenesis, including three-dimensional (3D) cell culture conditions generated by the cells themselves or by the use of biocompatible scaffolds.

Lee *et al*[53] developed a simplified procedure to reconstitute hair-producing skin. They obtained both epidermal and dermal cells from newborn mice, and mixed them in different ratios. A high density cellular suspension was prepared in drops of minimal volume on tissue culture inserts or wells. They allowed the cells to settle until gel consistency was obtained. Alternatively, they seeded the cells on the collagen-side of the Integra™ matrix (porous matrix of cross-linked bovine tendon collagen and glycosaminoglycan and a silicone layer). Both constructs were grafted in full thickness skin wounds generated on the back of athymic mice. After grafting, the epidermal cells formed a basal layer and some epidermal microcysts could be observed. Dermal cells started to form dermal condensations adjacent to the epidermal layer or cysts. Eight days after grafting hair germ started to appear and progressed to hair peg, observing complete hair follicles four days later that kept the ability to continuously cycle for at least one year. This method would allow preparing constructs with specific sizes and shapes useful to treat alopecia by tissue implants.

Two separate reports[54,55] showed hair follicle neogenesis using hair germs obtained by the previously described "organ germ method"[56]. One of them[54] generated bioengineered hair germs mixing epithelial and mesenchymal cells derived from mouse embryos within a collagen gel drop. In these culture conditions, the two cellular types generated a structure with two cellular layers separated by a translucent region they called hair germ. When these hair germs were transplanted ectopically into subrenal capsules, the presence of mature hair follicles was observed. These bioengineered hair follicles had a normal histological structure with the concentric epithelial layers of ORS and IRS, DP, hair matrix, and sebaceous glands. These follicular units transplanted intracutaneously in nude mice (Figure 1), were connected to the arrector pili muscle and nerve fibers and were able to produce hair shafts and cycling.

In addition, a fully functional orthotopic hair regeneration was demonstrated via intracutaneous transplantation of bioengineered hair follicle germs generated by epithelial and mesenchymal cells derived from mouse embryos[55] (Figure 1). These bioengineered hairs responded to hair cycles and had the correct structure of natural hair follicles and shafts, and the proper connections with surrounding host tissues. Qiao *et al*[57], using embryonic dermal cells and hair-peg-derived keratinocytes, generated cellular aggregates by hanging drop methods. These aggregates cultured in methylcellulose coated wells elongated, and after seven days, acquired hair follicle features. These structures termed "proto hairs", showed a morphological structure more developed respect the previously described "hair germs"[54,55]. They presented hair-like characteristics as an inner mass of cells similar to the DP structure surrounded by matrix-like keratinocytes and a partially keratinized substance that could represent intent to produce a hair shaft. After implantation into shallow incisions in nude mice (Figure 1), black-pigmented hair fibers were observed emerging from epidermis. These hairs persisted throughout the experimental period (6 mo) and were shown to regrow after plucking.

Although embryonic tissues are used as cellular sources, these studies showed the potentiality of dissociated cells to form hair follicle germs or proto-hairs able to generate mature hair follicles both by ectopic and orthotopic implantation. Collectively, these studies provide a proof of concept for the use of Follicular Cell Implantation to restore hair in patients suffering from hair loss.

As it was already mentioned, dermal cellular components from the skin, such as DPCs or dermal sheath cells (DSCs), have the potential to induce hair formation in *in vivo* assays and also induce *in vitro* differentiation of hair follicle stem cells to hair lineage[25,40]. Even if it is possible to isolate and culture DPCs, the inductive ability tends to fade with time in 2D cultures[6,58]. Considering that the hair follicle neogenesis requires a large number of DPCs, it is necessary to look for new strategies to maintain their inductive potential in culture in order to use them in skin bioengineering and hair follicle neogenesis.

The niche generated by the extracellular matrix (ECM) of DP probably plays a key role in keeping DPC inductive activity. Accordingly, the loss of ECM throughout culture time could be responsible for the

fading of DPC inductive properties.

DPCs are naturally aggregated at the hair-bulb base. In proper culture conditions, these cells tend to aggregate[13,59] and their inductive activity is preserved after they are grafted in this state. It has been demonstrated that it is possible to generate dermal papilla spheroidal microtissues using different methods including rotation, two-step rotation and flotation, and hanging drop[60-62]. Osada *et al*[63] artificially prepared DPC spheres by aggregation of mouse vibrissae follicle DPCs in a round-bottom 96-well low-binding plate. These spheres expressed higher amounts of versican, an indicator of inductive capability, than DPCs in monolayer cultures. In a "patch assay" (Figure 1), together with embryonic-epidermal cells, they induced hair follicle neogenesis, keeping their inductive capability for at least twenty-six passages.

However, these methods are labor intensive and the microtissues obtained have a very variable size and cell number content, which reduces reproducibility. DPCs can self-assemble into dense spheroids when seeded on controlled biomaterial surfaces such as poly (ethylene-co-vinyl alcohol) (EVAL)[64] or polyvinyl alcohol (PVA) membranes[65]. The DPC microtissues generated on EVAL surface[64] expressed DPC-markers such as neural cell adhesion molecule (NCAM)[66] and α-smooth muscle actin[67]. Alkaline phosphatase activity, which correlates with hair follicle inductive ability of DPC[68], was higher in spheroid microtissues than in monolayer cultured cells. Finally, the spheroids mixed with newborn mouse epidermal cells and injected into the hypodermis of nude mice in a "patch assay" (Figure 1) were able to generate new hair follicles.

The use of PVA as substratum material enhanced DPC aggregation[65], preventing cellular attachment and spreading. Using this material and reducing culture surface it is possible to control the number, size and compaction of spheroidal microtissues. DPCs quickly aggregated into a single spheroid whose diameter decreased progressively due to tissue compaction. The expression of DPC-inductive capability markers, versican[69] and alkaline phosphatase[68], were present in spheroids of various sizes. All these experiments were made both with adult DPCs from rat vibrissae, and human scalp hairs, giving similar results. The hypodermal injection in nude mice of eighty spheroids altogether with keratinocytes from newborn mice induced HF neogenesis. These results are very promising given that transplantation of dispersed cultured human DPCs had shown very low efficiency in inducing new hair follicles[70]. Using the same approach, heterotypic folliculoid microtissues or hair follicle-like organ germs were produced using dissociated adult epithelial and mesenchymal cells by self-assembly on EVAL coated wells[71]. Keratinocytes and DPCs seeded together grew into floating or loosely attached multicellular spheroids. Histological analysis showed that the spheroids are composed by a core of DPCs surrounded by keratinocytes. These keratinocytes expressed keratin 6, a cytokeratin preferentially expressed by ORS *in vivo*[72], and DPCs expressed versican, a marker associated to HF-inductive ability. Using "patch assays" (Figure 1) disperse DPCs and keratinocytes did not form new hair follicles, whereas heterotypic folliculoid microtissues did. These last experiments would contribute to the development of new strategies for scalable production of organoid microtissues of epithelial organs for hair follicle neogenesis by bioengineering approaches.

Altogether, these results strongly indicate that the hair-inductive capability of DPCs can be restored by three-dimensional spheroid cultures.

### *Hair follicle neogenesis using human cells*

During embryogenesis, dermal mesenchymal cells drive HF development and a condensate of these cells remains during adult life forming hair follicle DP. These cells retain the capability to induce hair follicle regeneration and neogenesis[21,73] as reviewed by Ohyama *et al*[5] and Yang *et al*[6]. However, most of these experiments were performed using murine and embryonic cells, whereas DPC from human origin showed many difficulties to maintain inductive capability in culture[74].

As discussed above, rat cultured DPCs self-aggregate within the dermis forming a papilla-like structure that synthesizes its own extracellular matrix[13]. However, such behavior was not observed with cultured human DPCs, highlighting specie-specific differences. Human keratinocytes from ORS and DPCs cultured in Matrigel™ (3D network of extracellular matrix proteins and collagen) have shown to form tubule-like structures in this skin-equivalent *in vitro*[75] and organize themselves into epidermoid cyst-like spheroids[72,76], but did not form complete hair follicles. Similarly, Sriwiriyanont *et al*[77], observed neofollicle formation in nude mice grafted with engineered skin substitutes containing murine DPC and human keratinocytes in a collagen-glycosaminoglycan matrix, but not in those containing human DPC and human keratinocytes.

Ehama *et al*[78] reported the formation of hair follicle-like structures using human primary cultures of foreskin- or adult-derived epidermal cells co-grafted with murine DPCs by a chamber assay (Figure 1). The innermost regions were similar to the hair cortex and medulla of mature human follicles. Hair shaft-like fibers occasionally emerged at the skin surface, and structures, reminiscent of the DP, at the follicle bottom were also observed. However, these structures showed no bulge region, nor all the follicular epithelial layers, and the DP-like tissue failed to express the anagen DP marker versican. These hair follicle-like structures correspond to the initial follicle formation suggesting that the differentiation process was altered.

Using the "flap assay"[52] (Figure 1), Qiao *et al*[79] showed that DPCs from human scalp combined with epidermis from mouse embryo produced mature

hair follicles that persisted and grew. The authors reported that the inductive potential of DPCs could be maintained using keratinocyte-conditioned medium. These DPCs were able to form DP and dermal sheath *in vivo*.

Spheroids microtissues were also obtaining by culturing human DPCs in a 96-well low-binding plate, and implanted intradermically into nude mice using the "patch assay" (Figure 1)[80]. This combination of human DPC spheres with murine neonatal epidermal cells generated new hair follicles. In contrast, hair follicles were never observed when monolayer DPC cultures were used.

Cultured human DPCs do not induce hair neogenesis unless changes in the culture conditions are made. In this sense, the formation of DPC-microsphere tissues has shown to improve hair-inductive properties of rodent cells as well as the expression of cell-markers associated with these properties[63-65]. Therefore, all these observations encouraged investigators to look for new strategies intended to achieve the first step toward human hair follicle neogenesis that is, providing culture conditions that confer hair-inductive ability to human DPCs. Higgins *et al*[74] evaluated the human DPC-transcriptome observing that monolayer DPC cultures showed the most important changes immediately after early outgrowths from DP explants. These changes involved 3729 transcripts, including several involved in hair follicle development. This large number of differentially expressed transcripts shows the physiological changes that 2D culture conditions do on the molecular signature of DPCs. The generation of DPC spheroids by the hanging drop method partially restores the intact DP signature. These human-DPC spheres induced hair follicle neogenesis when placed in between foreskin epidermis and dermis and grafted onto nude mice (Figure 1). These hair follicles showed a DP and dermal sheath highly positive for alkaline phosphatase, hair specific keratin markers disposed in concentric layers and blood vessel formation around follicles. Nevertheless, neither sebaceous glands nor hair fibers emerging from the skin surface were seen. These results suggested that human DPC spheroids would be able to initiate hair follicle morphogenesis but the production of a complete hair follicle requires additional signals.

Recently, Miao *et al*[81] produced DPC-spheroids from cultured DPCs on a Matrigel™ scaffold. As reported by Higgins *et al*[74], the DPC spheres were shown to restore DP-signature gene expression of NCAM, versican and α-smooth muscle actin (α-SMA), lost during monolayer culture. The authors also claimed that these DPC-spheres, combined with hair germinal matrix cells, onto Matrigel-coated plates produced colorless fiber-like structures *in vitro*.

Other alternative to culture dermal mesenchymal cells in 3D conditions is the organotypic method consisting in culturing cells inside a scaffold. Using this method, Wu *et al*[82] prepared a collagen gel with human DPCs or dermal sheath cells (DSCs). This gel was seeded with keratinocytes from interfollicular skin, superior ORS or inferior ORS obtaining in vitro a bilayered skin. Nonetheless, only the constructs containing superior ORS keratinocytes showed hair follicle-like structures. That is, an outer root sheath, nearly ten layers of concentric epithelial cells, a middle layer IRS-like and some keratinoid substances in the center, similar to a hair fiber. When these constructs were transplanted into nude mice (Figure 1), only the organotypic cultured tissue containing superior ORS keratinocytes and DPCs exhibited hair follicle structures.

In a recent study[83], composite skin substitutes were generated by seeding human neonatal foreskin keratinocytes onto a dermal equivalent structure cultured in air-liquid interface condition. This dermal equivalent was composed of DPCs from human scalp contained in a collagen-I gel. Eight weeks after grafted onto nude mice (Figure 1), these constructs presented hair follicles showing bulb, dermal sheath, hair matrix and cortex. Histological analysis showed concentric layers of IRS and ORS, sebaceous glands and hair shaft. Inmuno-histochemistry assays revealed that both epithelial and dermal cells from neo-follicles were from human origin, and that DPCs and DSCs expressed human nestin and versican.

The analysis of all these reports leads to the conclusion that hair follicle neogenesis using human epithelial and dermal cells is a very difficult task that requires special culture conditions, somehow recreating the normal or embryonic skin environment, and the use of embryonic or neonatal cells.

Very recently, we prepared in our laboratory a dermal-epidermal skin substitute by seeding an acellular dermal matrix with cultured hair follicle epithelial stem cells and DPCs, both obtained from adult human scalp[84]. These constructs were grafted into a full-thickness wound generated on nude mice skin (Figure 1). In only fourteen days, histological structures reminiscent of many different stages of embryonic hair follicle development were observed in the grafted area. These structures showed concentric cellular layers of human origin, and expressed k6hf, a keratin present in epithelial cells of the companion layer. Although the presence of fully mature hair follicles was not observed, these results represent, up to our knowledge, the first report showing that both epithelial and dermal cultured cells from adult human scalp in a dermal scaffold were able to produce *in vivo* structures that recapitulate embryonic hair development.

## ANALYSIS OF PATENTS TRENDS IN HAIR FOLLICLE REGENERATION AND NEOGENESIS

So far, we have made a concise review of the scientific

literature describing the attempts to regenerate the hair follicle. We have also mentioned a number of reviews on academic articles dealing with hair regeneration or wound induced follicle neogenesis that have been published in recent years[85]. However, reviews on tissue engineering and hair regeneration patents are less common. Many of these strategies have been combined in different methods intended for use in active applications. In this section, we review and analyze published patents on hair regeneration[86-150], as a measure of the interest in the industry for this area of research, identifying the currently available technical developments, favorite research strategies and main points of interest. Parameters analyzed include not only chronological patent publishing trends, but also the most cited patents, top patent owners (assignees), most cited documents, and the classification of patents among technology areas according to the International Patent Classification (IPC). Moreover, hair regeneration applications that have been patented throughout the years are screened (Figure 2).

## LITERATURE STUDY

Patent search was performed using the Thomson Innovation Database, a collaboaration platform for searching and analyzing global patents, integrated with analytics and workflow tools which allow access to more than 40 databases from different countries. For each particular group of hair regeneration, specific keywords (descriptors) were selected. Descriptors were chosen based on the synergy of two approaches: the search of specific keywords and concepts used in academic publications, and the selection of additional descriptors from specific patent vocabulary. Therefore, the keywords are: Stem cell; progenitor cell; precursor cell; repair; growth; wound; regeneration; neogenesis; skin; tissue engineering; dermis; epidermis; dermal; hair follicle; sebaceous gland and sebaceous unit. The presence of the chosen descriptors was checked in the title, abstract and claims of existing patents, considering documents published between years 1990 and 2014. More than forty technology patent and patent application databases were used for patent search, including the Spanish OEPM, the American USPTO, the worldwide WIPO, the European EPO, the patent offices of France, Germany, Great Britain and the Far-Eastern patent offices of Japan, China and South Korea. Besides the keywords, the search process was completed with the use of the IPC codes under which the patents of interest could be framed. The combination of the search approaches previously mentioned offered a universe of about 475 patent families, which were manually reviewed and filtered, setting aside those directed to gene therapies, mesenchymal, embryonic stem cell, osteoblast, neuron and hematopoietic cell and others. The result of this process was the identification of 65 patent families[86-150]. The selection of the document to be considered in patent trends was the first published. It is important to note that patent applications can be published in different countries under different national numbers. This is called the family of the patent. In the search performed for the present study, care was taken to include just one patent per family.

Tables showing patents that are most frequently cited take into account all the patents in the family, since it may occur that the earliest document is not necessarily the most cited one.

### *Competitive dynamics*

The competitive dynamics indicator shows the distribution of patents among the different organizations, providing a general picture of the tissue repair regeneration industry and their relative positioning. On the basis of this information we can identify the most prolific applicants for hair regeneration technology patents in the world, by establishing the number of applications filed by each company.

Table 1 lists the applicants that have filed more than one application, which will be our "Reference Group". Most of the members of this group are manufacturing companies, indicating that this technology belongs to the industrial sector. The highest percentile is shared by a small number of applicants with five patents each, while a second group includes a wide range of companies with no clear technological leadership.

The group of companies of the first percentile shown in Table 1, are the leading hair regeneration manufacturers Shiseido Co ltd and Aderans Research Institute, which own more than five published patents each, and which own almost 20% of all filed applications in this field.

The second percentile of institutions having one or two applications each could offer a favorable scenario for technology transfer.

It should be highlighted that there are over 20 institutions or about 40% patent applications from non-profit institutions including universities and foundations. The most prolific institutions are: Jilin University, University of Pennsylvania, University of Southern California and National Taiwan University, which gives an idea that this field is very prolific in academic institutions.

### *Technology trends*

The patent technology trends indicator introduces the serial number of patent applications showing how technology has evolved over time as well as its appropriation by companies and non-profit institutions in the field of hair regeneration. This technological area has experienced constant growth. In the 90's an average of 2.5 patent applications were filed per year, and in the first decade of the 21$^{th}$ century the average increased to 6 patent applications per year (Figure 2).

The years 2011 and 2012 stand out as the ones with the most patent applications, 10 and 16 respectively.

**Table 1 Owners of hair regeneration patents and patent applications matching all the descriptors found in academic literature**

| Assignee | No of patent applications | Percentage |
|---|---|---|
| Shiseido Co Ltd | 5 | 7.69% |
| Aderans Research Institute Inc | 5 | 7.69% |
| Hospital for Sick Children | 2 | 3.08% |
| Biointegrence Inc, phoenixbio Co Ltd | 2 | 3.08% |
| Follica Inc | 2 | 3.08% |
| Organ technologies Inc | 2 | 3.08% |
| Universitiy of Jilin | 2 | 3.08% |
| University of Southern California | 2 | 3.08% |
| National University of Taiwan | 2 | 3.08% |
| Aderans Research Institute Inc, Bioamide Inc | 1 | 1.54% |
| Agency for science, technology and research | 1 | 1.54% |
| Alvi Armani Genomics Inc | 1 | 1.54% |
| Anticancer Inc, Li L, Yang M | 1 | 1.54% |
| Beijing Yonghe Hair Transplant Technology | 1 | 1.54% |
| Biomaster KK | 1 | 1.54% |
| Chen B, Gao Q, Zeng Q | 1 | 1.54% |
| Chinese Academy of Science Institute of Zoology | 1 | 1.54% |
| Total No of patent applications | 65 | 100.00% |
| No of Entities | 50 | |

Own investigation using patent data base Thomson Innovation.

Aderans Corp Company filed most patent applications from 2005 to 2008. Shiseido and Follica, as well as the University of Pennsylvania have filed patent applications in recent years.

From this general perspective, it can be inferred that the technological field is, at present time, in a developmental stage, with an annual growth rate of 10%, based on the number of patent documents filed since 2010.

From another perspective, Figure 3 shows the cumulative patent families published in the last 20 years. As it can be seen, since 2005 there has been a remarkable increase of attraction in the area: while 50% of the patents required 15 years to be generated, the remaining 50% has been filed over the past five years.

The analysis of patent application-trends shows that, at the present time, the development of hair regeneration technology is evolving positively, making this technological field attractive to companies that start producing and selling products as a result of the development of hair regeneration technology.

### *Technological leadership indicator*

The technology leadership indicator identifies specific companies with the most consolidated and developed technologies in this specific field. The Patents which have received the highest number of citations are considered to have the highest degree of technological progress or impact. The analysis of patent citations allows the identification of patents which have been most useful for later researchers and for the development of newer technologies and may illustrate the diffusion of technical knowledge and its different practical applications throughout the years.

Just as those patents that have received most citations are not always the most relevant ones, and those most relevant are not always the most cited ones; a number of empirical studies demonstrate a strong correlation between those variables. In Table 2 the 10 most cited institutions as references are listed.

Up to the present, New York University and Pennsylvania State University have received the highest number of citations with 29 citations which reflects the importance of the works of Lavker *et al*[86] previously described. Following these institutions, companies likse Biointegrence Co and Phoenixbio Co Inc, and Anticancer Inc have 17 citations. The Hospital for Sick Children, Aderans Research Institute Inc, Sci Torico Innovations Inc and Trichoscience Innovations Inc are among the most innovative companies.

Skin substitutes were among the earliest products to be developed using principles of tissue engineering, and their success is evident in the clinical use of several commercially available products. However, skin substitutes capable of performing all the functions of normal skin are not currently available, which limits their use in patients. Hair follicle neogenesis is not observed using current skin substitutes. Thus, the efforts in hair and skin bioengineering continue to be in a leading place in regenerative medicine. Furthermore, hair restoration is one of the fastest growing areas of cosmetic therapies for both men and women.

Hair follicles are important not only for appearance, skin hydration, barrier formation, and protection against pathogens but also in wound healing. As hair follicles store epidermal stem cells, skin with hair follicles heals faster. In addition, bulge stem cells are less susceptible to loss through minor trauma and damage through ultraviolet light. Thus, treatments that involve neogenesis of normal hair follicles would find much wider application for restoring normal skin

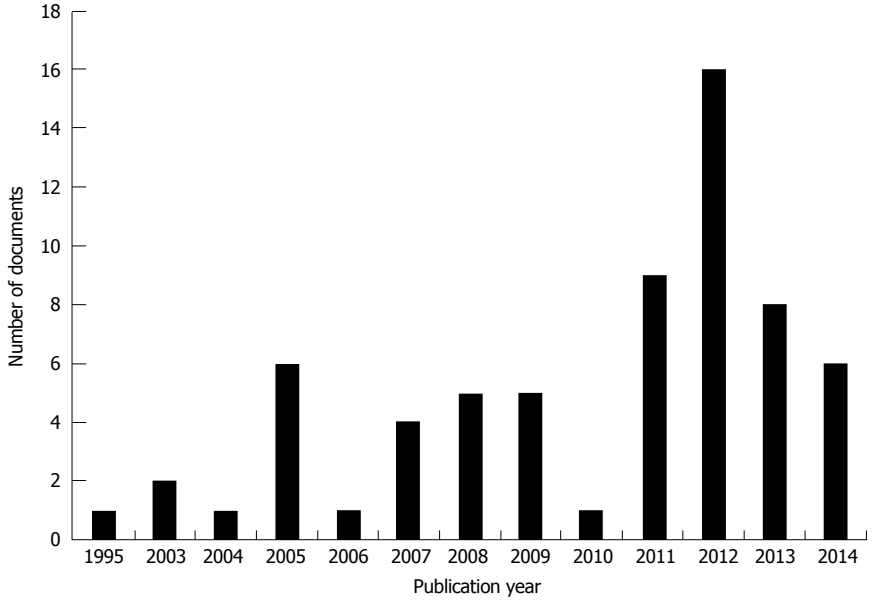


**Figure 2 Evolution of the number of patent applications related to hair regeneration (time frame 1990-2014).** Own investigation using patent data base Thomson Innovation.

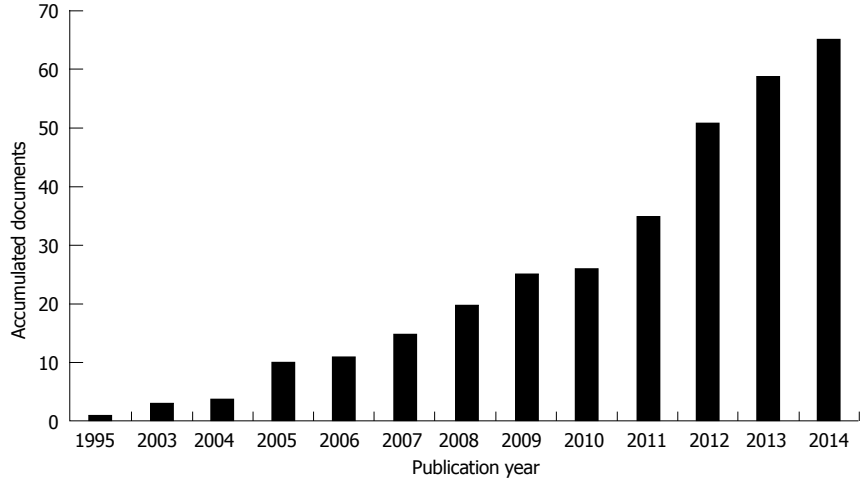


**Figure 3 Evolution of accumulated patent families.** Tendency line. Own generation based on patent databases.

function and appearance.

Methods and compositions capable of generating morphologically-correct, fully-developed human hair follicles, useful for treating conditions such as full- or partial-thickness skin loss, wounds, burns, scars, and hair loss have been developed in order to fulfill this necessity. In this analysis, only patents aiming to hair follicle regeneration or neogenesis have been selected. As reviewed, it has been determined that both epithelial cells and mesenchymal cells are essential for hair follicle regeneration. The regeneration of chimeric hair follicles comprised of mouse DPC and human epithelial cells has been shown to be possible, however it is still not possible to regenerate completely human hair follicles. One of the reasons for this is the difficulty to obtain an adequate number of human DPCs having the ability to induce hair follicle formation to be used for transplantation. Consequently, most of the patents filed in this field provide cellular compositions capable of hair neogenesis and regeneration or methods for isolating progenitor skin cells as well as the culture conditions that will allow them to keep their inductive properties to promote hair neogenesis or regeneration.

The first most cited patent listed in Table 2 (WO1995001423), published in 1995, described a method of culturing and modulating subpopulations of follicular keratinocytes from upper portions of hair follicle to be used for identifying agents which stimulate hair growth or prevent hair loss and useful for follicular reconstruction and transplantation as well as wound coverage in burns and skin ulcer[86] .

In 2005 a patent application (WO2005053763A1) developed a method for transplanting the dermal papilla or dermal papilla cells and outer skin epidermis

**Table 2 Most cited patents from 1990 to present**

| Publication number | Title | Assignee | Inventor | Publication year | Count of citing patents | Ref. |
|---|---|---|---|---|---|---|
| WO1995001423A1 | Methods of culturing and modulating the growth of hair follicular stem cells | New York State Univ, Pennsylvania Univ | Lavker RM, Sun T, Yang J | 1995 | 29 | [86] |
| WO2005053763A1 | Hair growth method | Biointegrence Inc, Phoenixbio Co Ltd | Matsunaga M, Matsunaga MC, Shimada T, Shimada TC, Toyoshima K, Toyoshima KC, Yoshizato K | 2005 | 17 | [94] |
| WO2003024406A2 | Nestin-expressing hair follicle stem cells | Anticancer Inc | Li L, Lingna L, Meng Y, Ri R, Yang M | 2003 | 17 | [87] |
| WO2005071063A1 | Methods of making and using skin-derived stem cells | Hospital For Sick Children | Biernaskie J, Fernandes K, Fernandez K, Mckenzie I, Miller F, Miller FD | 2005 | 16 | [91] |
| WO2002060396A2 | Hair follicle neogenesis by injection of follicle progenitor cells | Aderans Res Inst Inc, Bioamide Inc | Barrows TH | 2002 | 16 | [88] |
| US20050214344A1 | Tissue engineered biomimetic hair follicle graft | Aderans Res Inst Inc | Barrows T, Barrows TH, Cochran S, Cochran SA, Marshall B | 2005 | 15 | [90] |
| US20070092496A1 | Method of delivering cells to the skin | Aderans Res Inst Inc | Du X, Stenn K, Stenn KS, Washenik K, Washenik KJ, Zhang Y, Zheng Y | 2007 | 10 | [99] |
| WO2003104443A2 | Hair follicle mesenchymal stem cells and use thereof | Torico Sci Innovations Inc, Trichoscience Innovations Inc | Hoffmann R, Mcelwee KJ | 2003 | 9 | [89] |
| US20070122387A1 | Hair grafts derived from plucked hair | Aderans Res Inst Inc | Barrows TH, Cochran SA, Marshall B, Schlicher R, Su Y | 2007 | 9 | [105] |
| WO2007062386A2 | Hair follicle graft from tissue engineered skin | Aderans Res Inst Inc | Barrows TH, Macintyre P, Washenik KJ | 2007 | 8 | [98] |

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tissue or epidermal cells to hairless scalp to regenerate hair[94].

Different tissue engineered-hair follicle grafts were also patented; a scaffold constructed to mimic the architecture of the native hair follicle and for percutaneous implantation facilitating the follicle neogenesis process[90] (US20050214344), a hair graft comprising plucked hairs having adhered epidermal stem cells and follicular dermal cells for implantation into wounded skin[105] (US20070122387A1) and a hair graft comprising a scaffold, and tissue engineered epidermal and dermal layer with hair follicle progenitor cells (WO2007062386A2)[98].

Several methods for isolating skin-derived precursor stem cells from hair follicles or dermal papillae as well as the culture conditions or the delivery into the skin of these cells in order to induce hair growth are also among the most cited patents (WO2002060396A, WO2005071063A1 and US20070092496A1)[88,91,99].

Finally, among the most cited patents are those describing methods for isolating hair follicle mesenchymal stem cells useful for treating alopecia and gene therapy (WO2003104443A2)[89] or useful for hair loss, burns or skin replacement as well as for treating neurological or degenerative disorders (WO2003-024406A2)[87].

The rest of the patents analyzed whose content is not mentioned, can be found in the References section[86-150] at the end of this review.

## CONCLUSION

Although a successful strategy to generate human hair follicles from adult cells has not yet been reported, the results presented in this review suggest the issues that need to be addressed before success can be achieved. Perhaps the most important of those issues is to provide cells with a three-dimensional structure that simulates the natural scaffold. Herein the cells should develop and interact among themselves or with other cell types in the epithelial-mesenchymal interactions. Improving culture conditions that allow the expansion of these cells without losing their natural properties, as well as selecting the appropriate epithelial stem cells, should give us the tools needed to face the challenge of regenerating human hair follicles.

These efforts in hair and skin bioengineering are also visible in the growing number of patent applications filed during the last decade, indicating that this technological field is not only attractive to academic research but also to the companies that own almost half of these patents.

## REFERENCES

1 **Papini R**. Management of burn injuries of various depths. *BMJ* 2004; **329**: 158-160 [PMID: 15258073 DOI: 10.1136/bmj.329.7458.158]

2 **Shevchenko RV**, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. *J R*

*Soc Interface* 2010; **7**: 229-258 [PMID: 19864266 DOI: 10.1098/rsif.2009.0403]

3 **Blanpain C**, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 2004; **118**: 635-648 [PMID: 15339667 DOI: 10.1016/j.cell.2004.08.012]

4 **Oshima H**, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* 2001; **104**: 233-245 [PMID: 11207364 DOI: 10.1016/S0092-8674(01)00208-2]

5 **Ohyama M**, Zheng Y, Paus R, Stenn KS. The mesenchymal component of hair follicle neogenesis: background, methods and molecular characterization. *Exp Dermatol* 2010; **19**: 89-99 [PMID: 19650868 DOI: 10.1111/j.1600-0625.2009.00935.x]

6 **Yang CC**, Cotsarelis G. Review of hair follicle dermal cells. *J Dermatol Sci* 2010; **57**: 2-11 [PMID: 20022473 DOI: 10.1016/j.jdermsci.2009.11.005]

7 **Martin P**. Wound healing--aiming for perfect skin regeneration. *Science* 1997; **276**: 75-81 [PMID: 9082989 DOI: 10.1126/science.276.5309.75]

8 **Paus R**, Foitzik K. In search of the "hair cycle clock": a guided tour. *Differentiation* 2004; **72**: 489-511 [PMID: 15617561 DOI: 10.1111/j.1432-0436.2004.07209004.x]

9 **Otberg N**, Richter H, Schaefer H, Blume-Peytavi U, Sterry W, Lademann J. Variations of hair follicle size and distribution in different body sites. *J Invest Dermatol* 2004; **122**: 14-19 [PMID: 14962084 DOI: 10.1046/j.0022-202X.2003.22110.x]

10 **Schmidt-Ullrich R**, Paus R. Molecular principles of hair follicle induction and morphogenesis. *Bioessays* 2005; **27**: 247-261 [PMID: 15714560 DOI: 10.1002/bies.20184]

11 **Stenn KS**, Paus R. Controls of hair follicle cycling. *Physiol Rev* 2001; **81**: 449-494 [PMID: 11152763]

12 **Millar SE**. Molecular mechanisms regulating hair follicle development. *J Invest Dermatol* 2002; **118**: 216-225 [PMID: 11841536 DOI: 10.1046/j.0022-202x.2001.01670.x]

13 **Jahoda CA**, Oliver RF. Vibrissa dermal papilla cell aggregative behaviour in vivo and in vitro. *J Embryol Exp Morphol* 1984; **79**: 211-224 [PMID: 6716045]

14 **Christiano AM**. Epithelial stem cells: stepping out of their niche. *Cell* 2004; **118**: 530-532 [PMID: 15339656 DOI: 10.1016/j.cell.2004.08.024]

15 **Tumbar T**, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. Defining the epithelial stem cell niche in skin. *Science* 2004; **303**: 359-363 [PMID: 14671312 DOI: 10.1126/science.1092436]

16 **Morris RJ**, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G. Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 2004; **22**: 411-417 [PMID: 15024388 DOI: 10.1038/nbt950]

17 **Ito M**, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, Cotsarelis G. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 2005; **11**: 1351-1354 [PMID: 16288281 DOI: 10.1038/nm1328]

18 **Nowak JA**, Polak L, Pasolli HA, Fuchs E. Hair follicle stem cells are specified and function in early skin morphogenesis. *Cell Stem Cell* 2008; **3**: 33-43 [PMID: 18593557 DOI: 10.1016/j.stem.2008.05.009]

19 **Botchkarev VA**, Kishimoto J. Molecular control of epithelial-mesenchymal interactions during hair follicle cycling. *J Investig Dermatol Symp Proc* 2003; **8**: 46-55 [PMID: 12894994 DOI: 10.1046/j.1523-1747.2003.12171.x]

20 **Fuchs E**, Merrill BJ, Jamora C, DasGupta R. At the roots of a never-ending cycle. *Dev Cell* 2001; **1**: 13-25 [PMID: 11703920 DOI: 10.1016/S1534-5807(01)00022-3]

21 **Gharzi A**, Reynolds AJ, Jahoda CA. Plasticity of hair follicle dermal cells in wound healing and induction. *Exp Dermatol* 2003; **12**: 126-136 [PMID: 12702140 DOI: 10.1034/j.1600-0625.2003.00106.x]

22 **Jahoda CA**, Reynolds AJ. Hair follicle dermal sheath cells: unsung participants in wound healing. *Lancet* 2001; **358**: 1445-1448 [PMID: 11705511 DOI: 10.1016/S0140-6736(01)06532-1]

23 **Alonso L**, Fuchs E. The hair cycle. *J Cell Sci* 2006; **119**: 391-393 [PMID: 16443746 DOI: 10.1242/jcs02793]

24 **Greco V**, Chen T, Rendl M, Schober M, Pasolli HA, Stokes N, Dela Cruz-Racelis J, Fuchs E. A two-step mechanism for stem cell activation during hair regeneration. *Cell Stem Cell* 2009; **4**: 155-169 [PMID: 19200804 DOI: 10.1016/j.stem.2008.12.009]

25 **Roh C**, Tao Q, Lyle S. Dermal papilla-induced hair differentiation of adult epithelial stem cells from human skin. *Physiol Genomics* 2004; **19**: 207-217 [PMID: 15292489 DOI: 10.1152/physiolgenomics.00134.2004]

26 **Hsu YC**, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. *Cell* 2011; **144**: 92-105 [PMID: 21215372 DOI: 10.1016/j.cell.2010.11.049]

27 **Schneider MR**, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. *Curr Biol* 2009; **19**: R132-R142 [PMID: 19211055 DOI: 10.1016/j.cub.2008.12.005]

28 **Kligman AM**, Strauss JS. The formation of vellus hair follicles from human adult epidermis. *J Invest Dermatol* 1956; **27**: 19-23 [PMID: 13357817 DOI: 10.1038/jid.1956.71]

29 **Cotsarelis G**, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 1990; **61**: 1329-1337 [PMID: 2364430 DOI: 10.1016/0092-8674(90)90696-C]

30 **Morris RJ**, Potten CS. Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. *J Invest Dermatol* 1999; **112**: 470-475 [PMID: 10201531 DOI: 10.1046/j.1523-1747.1999.00537.x]

31 **Ito M**, Kizawa K, Toyoda M, Morohashi M. Label-retaining cells in the bulge region are directed to cell death after plucking, followed by healing from the surviving hair germ. *J Invest Dermatol* 2002; **119**: 1310-1316 [PMID: 12485433 DOI: 10.1046/j.1523-1747.2002.19644.x]

32 **Plikus MV**, Mayer JA, de la Cruz D, Baker RE, Maini PK, Maxson R, Chuong CM. Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. *Nature* 2008; **451**: 340-344 [PMID: 18202659 DOI: 10.1038/nature06457]

33 **Festa E**, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M, Horsley V. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. *Cell* 2011; **146**: 761-771 [PMID: 21884937 DOI: 10.1016/j.cell.2011.07.019]

34 **Kishimoto J**, Burgeson RE, Morgan BA. Wnt signaling maintains the hair-inducing activity of the dermal papilla. *Genes Dev* 2000; **14**: 1181-1185 [PMID: 10817753 DOI: 10.1101/gad.14.10.1181]

35 **Chen CC**, Chuong CM. Multi-layered environmental regulation on the homeostasis of stem cells: the saga of hair growth and alopecia. *J Dermatol Sci* 2012; **66**: 3-11 [PMID: 22391240 DOI: 10.1016/j.jdermsci.2012.02.007]

36 **Randall VA**, Hibberts NA, Thornton MJ, Merrick AE, Hamada K, Kato S, Jenner TJ, de Oliveira I, Messenger AG. Do androgens influence hair growth by altering the paracrine factors secreted by dermal papilla cells? *Eur J Dermatol* 2001; **11**: 315-320 [PMID: 11399537]

37 **Inui S**, Fukuzato Y, Nakajima T, Yoshikawa K, Itami S. Androgen-inducible TGF-beta1 from balding dermal papilla cells inhibits epithelial cell growth: a clue to understand paradoxical effects of androgen on human hair growth. *FASEB J* 2002; **16**: 1967-1969 [PMID: 12397096 DOI: 10.1096/fj.02-0043fje]

38 **Kwack MH**, Sung YK, Chung EJ, Im SU, Ahn JS, Kim MK, Kim JC. Dihydrotestosterone-inducible dickkopf 1 from balding dermal papilla cells causes apoptosis in follicular keratinocytes. *J Invest Dermatol* 2008; **128**: 262-269 [PMID: 17657240 DOI: 10.1038/sj.jid.5700999]

39 **Grisouard J**, Mayer D. Specific involvement of glycogen synthase kinase-3 in the function and activity of sex steroid hormone receptors reveals the complexity of their regulation. *J Steroid Biochem Mol Biol* 2009; **117**: 87-92 [PMID: 19703560 DOI: 10.1016/j.jsbmb.2009.08.002]

40 **Leirós GJ**, Attorresi AI, Balañá ME. Hair follicle stem cell differentiation is inhibited through cross-talk between Wnt/β-catenin and androgen signalling in dermal papilla cells from patients with

androgenetic alopecia. *Br J Dermatol* 2012; **166**: 1035-1042 [PMID: 22283397 DOI: 10.1111/j.1365-2133.2012.10856.x]
41 **Valente Duarte de Sousa IC**, Tosti A. New investigational drugs for androgenetic alopecia. *Expert Opin Investig Drugs* 2013; **22**: 573-589 [PMID: 23550739 DOI: 10.1517/13543784.2013.784743]
42 **Cohen J**. The transplantation of individual rat and guineapig whisker papillae. *J Embryol Exp Morphol* 1961; **9**: 117-127 [PMID: 13694386]
43 **Oliver RF**. The induction of hair follicle formation in the adult hooded rat by vibrissa dermal papillae. *J Embryol Exp Morphol* 1970; **23**: 219-236 [PMID: 4926619]
44 **Oliver RF**. Ectopic regeneration of whiskers in the hooded rat from implanted lengths of vibrissa follicle wall. *J Embryol Exp Morphol* 1967; **17**: 27-34 [PMID: 6040555]
45 **Horne KA**, Jahoda CA. Restoration of hair growth by surgical implantation of follicular dermal sheath. *Development* 1992; **116**: 563-571 [PMID: 1289054]
46 **Reynolds AJ**, Lawrence C, Cserhalmi-Friedman PB, Christiano AM, Jahoda CA. Trans-gender induction of hair follicles. *Nature* 1999; **402**: 33-34 [PMID: 10573414]
47 **Jahoda CA**. Induction of follicle formation and hair growth by vibrissa dermal papillae implanted into rat ear wounds: vibrissa-type fibres are specified. *Development* 1992; **115**: 1103-1109 [PMID: 1451660]
48 **Lichti U**, Weinberg WC, Goodman L, Ledbetter S, Dooley T, Morgan D, Yuspa SH. In vivo regulation of murine hair growth: insights from grafting defined cell populations onto nude mice. *J Invest Dermatol* 1993; **101**: 124S-129S [PMID: 8326145]
49 **Prouty SM**, Lawrence L, Stenn KS. Fibroblast-dependent induction of a murine skin lesion with similarity to human common blue nevus. *Am J Pathol* 1996; **148**: 1871-1885 [PMID: 8669473]
50 **Zheng Y**, Du X, Wang W, Boucher M, Parimoo S, Stenn K. Organogenesis from dissociated cells: generation of mature cycling hair follicles from skin-derived cells. *J Invest Dermatol* 2005; **124**: 867-876 [PMID: 15854024 DOI: 10.1111/j.0022-202X.2005.23716.x]
51 **Gilmour SK**, Teti KA, Wu KQ, Morris RJ. A simple in vivo system for studying epithelialization, hair follicle formation, and invasion using primary epidermal cells from wild-type and transgenic ornithine decarboxylase-overexpressing mouse skin. *J Invest Dermatol* 2001; **117**: 1674-1676 [PMID: 11886542 DOI: 10.1046/j.0022-202x.2001.01597.x]
52 **Qiao J**, Philips E, Teumer J. A graft model for hair development. *Exp Dermatol* 2008; **17**: 512-518 [PMID: 18070082 DOI: 10.1111/j.1600-0625.2007.00661.x]
53 **Lee LF**, Jiang TX, Garner W, Chuong CM. A simplified procedure to reconstitute hair-producing skin. *Tissue Eng Part C Methods* 2011; **17**: 391-400 [PMID: 21034159 DOI: 10.1089/ten.tec.2010.0477]
54 **Asakawa K**, Toyoshima KE, Ishibashi N, Tobe H, Iwadate A, Kanayama T, Hasegawa T, Nakao K, Toki H, Noguchi S, Ogawa M, Sato A, Tsuji T. Hair organ regeneration via the bioengineered hair follicular unit transplantation. *Sci Rep* 2012; **2**: 424 [PMID: 22645640 DOI: 10.1038/srep00424]
55 **Toyoshima KE**, Asakawa K, Ishibashi N, Toki H, Ogawa M, Hasegawa T, Irié T, Tachikawa T, Sato A, Takeda A, Tsuji T. Fully functional hair follicle regeneration through the rearrangement of stem cells and their niches. *Nat Commun* 2012; **3**: 784 [PMID: 22510689 DOI: 10.1038/ncomms1784]
56 **Nakao K**, Morita R, Saji Y, Ishida K, Tomita Y, Ogawa M, Saitoh M, Tomooka Y, Tsuji T. The development of a bioengineered organ germ method. *Nat Methods* 2007; **4**: 227-230 [PMID: 17322892 DOI: 10.1038/nmeth1012]
57 **Qiao J**, Turetsky A, Kemp P, Teumer J. Hair morphogenesis in vitro: formation of hair structures suitable for implantation. *Regen Med* 2008; **3**: 683-692 [PMID: 18729793 DOI: 10.2217/17460751.3.5.683]
58 **Paus R**, Cotsarelis G. The biology of hair follicles. *N Engl J Med* 1999; **341**: 491-497 [PMID: 10441606 DOI: 10.1056/NEJM199908123410706]
59 **Jahoda CA**, Horne KA, Oliver RF. Induction of hair growth by implantation of cultured dermal papilla cells. *Nature* 1984; **311**: 560-562 [PMID: 6482967 DOI: 10.1038/311560a0]
60 **Ihara S**, Watanabe M, Nagao E, Shioya N. Formation of hair follicles from a single-cell suspension of embryonic rat skin by a two-step procedure in vitro. *Cell Tissue Res* 1991; **266**: 65-73 [PMID: 1747915 DOI: 10.1007/BF00678712]
61 **Takeda A**, Matsuhashi S, Shioya N, Ihara S. Histodifferentiation of hair follicles in grafting of cell aggregates obtained by rotation culture of embryonic rat skin. *Scand J Plast Reconstr Surg Hand Surg* 1998; **32**: 359-364 [PMID: 9862102 DOI: 10.1080/02844319850158435]
62 **Kelm JM**, Fussenegger M. Microscale tissue engineering using gravity-enforced cell assembly. *Trends Biotechnol* 2004; **22**: 195-202 [PMID: 15038925 DOI: 10.1016/j.tibtech.2004.02.002]
63 **Osada A**, Iwabuchi T, Kishimoto J, Hamazaki TS, Okochi H. Long-term culture of mouse vibrissal dermal papilla cells and de novo hair follicle induction. *Tissue Eng* 2007; **13**: 975-982 [DOI: 10.1089/ten.2006.0304]
64 **Young TH**, Lee CY, Chiu HC, Hsu CJ, Lin SJ. Self-assembly of dermal papilla cells into inductive spheroidal microtissues on poly(ethylene-co-vinyl alcohol) membranes for hair follicle regeneration. *Biomaterials* 2008; **29**: 3521-3530 [PMID: 18533254 DOI: 10.1016/j.biomaterials.2008.05.013]
65 **Huang YC**, Chan CC, Lin WT, Chiu HY, Tsai RY, Tsai TH, Chan JY, Lin SJ. Scalable production of controllable dermal papilla spheroids on PVA surfaces and the effects of spheroid size on hair follicle regeneration. *Biomaterials* 2013; **34**: 442-451 [PMID: 23092862 DOI: 10.1016/j.biomaterials.2012.09.083]
66 **Müller-Röver S**, Peters EJ, Botchkarev VA, Panteleyev A, Paus R. Distinct patterns of NCAM expression are associated with defined stages of murine hair follicle morphogenesis and regression. *J Histochem Cytochem* 1998; **46**: 1401-1410 [PMID: 9815282 DOI: 10.1177/002215549804601209]
67 **Jahoda CA**, Reynolds AJ, Chaponnier C, Forester JC, Gabbiani G. Smooth muscle alpha-actin is a marker for hair follicle dermis in vivo and in vitro. *J Cell Sci* 1991; **99** (Pt 3): 627-636 [PMID: 1939373]
68 **McElwee KJ**, Kissling S, Wenzel E, Huth A, Hoffmann R. Cultured peribulbar dermal sheath cells can induce hair follicle development and contribute to the dermal sheath and dermal papilla. *J Invest Dermatol* 2003; **121**: 1267-1275 [PMID: 14675169 DOI: 10.1111/j.1523-1747.2003.12568.x]
69 **Kishimoto J**, Ehama R, Wu L, Jiang S, Jiang N, Burgeson RE. Selective activation of the versican promoter by epithelial-mesenchymal interactions during hair follicle development. *Proc Natl Acad Sci U S A* 1999; **96**: 7336-7341 [PMID: 10377415 DOI: 10.1073/pnas.96.13.7336]
70 **Soma T**, Fujiwara S, Shirakata Y, Hashimoto K, Kishimoto J. Hair-inducing ability of human dermal papilla cells cultured under Wnt/β-catenin signalling activation. *Exp Dermatol* 2012; **21**: 307-309 [PMID: 22417309 DOI: 10.1111/j.1600-0625.2012.01458.x]
71 **Yen CM**, Chan CC, Lin SJ. High-throughput reconstitution of epithelial-mesenchymal interaction in folliculoid microtissues by biomaterial-facilitated self-assembly of dissociated heterotypic adult cells. *Biomaterials* 2010; **31**: 4341-4352 [PMID: 20206989 DOI: 10.1016/j.biomaterials.2010.02.014]
72 **Havlickova B**, Bíró T, Mescalchin A, Tschirschmann M, Mollenkopf H, Bettermann A, Pertile P, Lauster R, Bodó E, Paus R. A human folliculoid microsphere assay for exploring epithelial- mesenchymal interactions in the human hair follicle. *J Invest Dermatol* 2009; **129**: 972-983 [PMID: 18923448 DOI: 10.1038/jid.2008.315]
73 **Hardy MH**. The secret life of the hair follicle. *Trends Genet* 1992; **8**: 55-61 [PMID: 1566372]
74 **Higgins CA**, Chen JC, Cerise JE, Jahoda CA, Christiano AM. Microenvironmental reprogramming by three-dimensional culture enables dermal papilla cells to induce de novo human hair-follicle growth. *Proc Natl Acad Sci USA* 2013; **110**: 19679-19688 [PMID: 24145441 DOI: 10.1073/pnas.1309970110]
75 **Chermnykh ES**, Vorotelyak EA, Gnedeva KY, Moldaver MV, Yegorov YE, Vasiliev AV, Terskikh VV. Dermal papilla cells induce

keratinocyte tubulogenesis in culture. *Histochem Cell Biol* 2010; **133**: 567-576 [PMID: 20336308 DOI: 10.1007/s00418-010-0691-0]

76 **Limat A**, Breitkreutz D, Hunziker T, Klein CE, Noser F, Fusenig NE, Braathen LR. Outer root sheath (ORS) cells organize into epidermoid cyst-like spheroids when cultured inside Matrigel: a light-microscopic and immunohistological comparison between human ORS cells and interfollicular keratinocytes. *Cell Tissue Res* 1994; **275**: 169-176 [PMID: 7509722]

77 **Sriwiriyanont P**, Lynch KA, Maier EA, Hahn JM, Supp DM, Boyce ST. Morphogenesis of chimeric hair follicles in engineered skin substitutes with human keratinocytes and murine dermal papilla cells. *Exp Dermatol* 2012; **21**: 783-785 [PMID: 23078401 DOI: 10.1111/exd.12003]

78 **Ehama R**, Ishimatsu-Tsuji Y, Iriyama S, Ideta R, Soma T, Yano K, Kawasaki C, Suzuki S, Shirakata Y, Hashimoto K, Kishimoto J. Hair follicle regeneration using grafted rodent and human cells. *J Invest Dermatol* 2007; **127**: 2106-2115 [PMID: 17429436 DOI: 10.1038/sj.jid.5700823]

79 **Qiao J**, Zawadzka A, Philips E, Turetsky A, Batchelor S, Peacock J, Durrant S, Garlick D, Kemp P, Teumer J. Hair follicle neogenesis induced by cultured human scalp dermal papilla cells. *Regen Med* 2009; **4**: 667-676 [PMID: 19761392 DOI: 10.2217/rme.09.50]

80 **Kang BM**, Kwack MH, Kim MK, Kim JC, Sung YK. Sphere formation increases the ability of cultured human dermal papilla cells to induce hair follicles from mouse epidermal cells in a reconstitution assay. *J Invest Dermatol* 2012; **132**: 237-239 [PMID: 21850026 DOI: 10.1038/jid.2011.250]

81 **Miao Y**, Sun YB, Liu BC, Jiang JD, Hu ZQ. Controllable production of transplantable adult human high-passage dermal papilla spheroids using 3D matrigel culture. *Tissue Eng Part A* 2014; **20**: 2329-2338 [PMID: 24528213 DOI: 10.1089/ten.TEA.2013.0547]

82 **Wu JJ**, Zhu TY, Lu YG, Liu RQ, Mai Y, Cheng B, Lu ZF, Zhong BY, Tang SQ. Hair follicle reformation induced by dermal papilla cells from human scalp skin. *Arch Dermatol Res* 2006; **298**: 183-190 [PMID: 16897077 DOI: 10.1007/s00403-006-0686-9]

83 **Thangapazham RL**, Klover P, Wang JA, Zheng Y, Devine A, Li S, Sperling L, Cotsarelis G, Darling TN. Dissociated human dermal papilla cells induce hair follicle neogenesis in grafted dermal-epidermal composites. *J Invest Dermatol* 2014; **134**: 538-540 [PMID: 23924901 DOI: 10.1038/jid.2013.337]

84 **Leirós GJ**, Kusinsky AG, Drago H, Bossi S, Sturla F, Castellanos ML, Stella IY, Balañá ME. Dermal papilla cells improve the wound healing process and generate hair bud-like structures in grafted skin substitutes using hair follicle stem cells. *Stem Cells Transl Med* 2014; **3**: 1209-1219 [PMID: 25161315 DOI: 10.5966/sctm.2013-0217]

85 **Chueh SC**, Lin SJ, Chen CC, Lei M, Wang LM, Widelitz R, Hughes MW, Jiang TX, Chuong CM. Therapeutic strategy for hair regeneration: hair cycle activation, niche environment modulation, wound-induced follicle neogenesis, and stem cell engineering. *Expert Opin Biol Ther* 2013; **13**: 377-391 [PMID: 23289545 DOI: 10.1517/14712598.2013.739601]

86 **Lavker RM**, Sun TT, Yang JS, inventors; New York State Univ, Pennsylvania Univ, assignees. Methods of culturing and modulating the growth of hair follicular stem cells. WO1995001423A1. 1995 Jan 12

87 **Li LN**, Yang M, inventors; AntiCancer Inc, assignee. Nestin-expressing hair follicle stem cells. WO2003024406A2. 2003 Mar 27

88 **Barrows** Thomas H, inventor; Aderans Res Inst Inc, Bioamide Inc, assignee. Hair follicle neogenesis by injection of follicle progenitor cells. WO2002060396A2. 2002 Aug 08

89 **Hoffmann R**, Mcelwee Kevin J, inventors; Torico Sci Innovations Inc, Trichoscience Innovations Inc, assignee. Hair follicle mesenchymal stem cells and use thereof. WO2003104443A2. 2003 Dec 18

90 Barrows Thomas H, Cochran Stephen A, Marshall B, inventors; Aderans Res Inst Inc, assignee. Tissue engineered biomimetic hair follicle graft. United States patent US20050214344A1. 2005 Sep 29

91 **Miller Freda D**, Fernandez K, Biernaskie J, Mckenzie I, inventors; Hospital For Sick Children, assignee. Methods of making and using skin-derived stem cells. WO2005071063A1. 2005 Aug 08

92 **Kitano M,** Shirakawa T, Tamaoki Tomoko, Sasahara Yusuke, inventors; Technology Seed Incubation KK, assignee. Cultured dermal cell, transplanting material and material for gene analysis using the same and method for preparation of cultured dermal cell. Japanese patent JP2005218445A. 2005 Aug 18

93 **Jahoda CAB**, Reynolds AJ, Whitehouse CJ, Hole N, inventors; Durham Univ, assignee. Differentiated cells. WO2005059119A3. 2005 Aug 11

94 **Yoshizato K**, Shimada T, Toyoshima K, Matsunaga M, inventors; Biointegrence Inc, Phoenixbio Co Ltd, assignees. Hair growth method. WO2005053763A1. 2005 Jun 16

95 **Kishimoto J**, Ehama R, Ideta R, Arai T, Yano K, Soma T, inventors; Shiseido Company Ltd, assignee. Method of preparing hair papilla cell preparation, composition and method for regenerating hair follicle and animal having regenerated hair follicle. WO2005033302A1. 2005 Apr 14

96 **Zheng Y**, Du XB, Wang W, Boucher M, Parimoo S, Stenn KS, inventors; Aderans Res Inst Inc, assignee. Organogenesis from dissociated cells. WO2006020958A2. 2006 Feb 23

97 **Li XL**, Zhang Y, Hua YQ, Guo Y, Zhang WH, Bai RX, inventors; Tianjin Hospital, assignee. Follicular stem cell originated from human hair and its amplifying preparation process. Hair follicle stem cell from human hair and method of proliferating and preparing it. Chinese patent CN101045916A. 2007 Oct 3

98 **Barrows Thomas H**, Macintyre P, Washenik KJ, inventors; Aderans Res Inst Inc, assignee. Hair follicle graft from tissue engineered skin. WO2007062386A2. 2007 May 31

99 **Zheng Y**, Du XB, Washenik K, Stenn KS, inventors; Aderans Res Inst Inc, assignee. Method of delivering hair follicle progenitor cells to the skin. United States patent US20070092496A1. 2007 Apr 26

100 **Toyoshima K**, Matsunaga M, Yoshizato K, inventors; Phoenixbio Co Ltd, Biointegrence Inc, assignees. Method for cultivation of hair follicular dermal sheath cell. WO2007037486A1. 2007 Apr 5

101 **Miller Freda D**, Biernaskie J, inventors; Hospital For Sick Children, assignee. Skin-derived precursor cells and uses thereof. WO2008148218A1. 2008 Dec 11

102 **Steinberg D**, Pojasek K, Prouty S, Cotsarelis G, Ito M, inventors; Follica Inc, Pennsylvania Univ, assignees. Methods, kits, and compositions for generating new hair follicles and growing hair. WO2008042216A2. 2008 Apr 10

103 **Kang KS**, Ra JC, inventors; Univ Seoul Nat Ind Found, Modecell Ltd, Rnl Bio Co Ltd, assignees. Method for isolation of a hair follicle stem cell and a composition for hair reproduction reproduction. WO2008004819A1. 2008 Jan 10

104 **Jung SH**, inventor; Everbridge Co Ltd, assignee. Method for culturing human hair follicle component cells, method for manufacturing bioartificial skin, and method for in vitro induction of hair structure using the cultured hair follicle component cells sheath cells, hair papilla cells, lower dermal sheath cells, melanocytes, and pilomatrix cells, a method for producing bioartificial skin from the cultured hair follicle component cells and a method for in vitro induction of hair follicle structure from the cultured hair follicle component cells. Korean patent KR2008071656A. 2008 Aug 5

105 **Cochran Stephen A**, Marshall B, Barrows Thomas H, Su YD, Schlicher R, inventors; Aderans Res Inst Inc, assignee. Hair grafts derived from plucked hair. United States patent US20070122387A1. 2007 May 31

106 **Lindner G**, Lauster R, inventors; Berlin Tech Univ, assignee. Methods for producing hair microfollicles and de novo papillae and their use for in vitro tests and in vivo implantations. WO2009118283A1. 2009 Oct 1

107 **Philips EJ**, Qiao J, Teumer J, inventors; Intercytex Ltd, assignee. Cell co-culture for hair follicle production. United States patent US20090198336A1. 2009 Aug 6

108 **Fujiwara S**, Kishimoto J, Soma T, inventors; Shiseido Company Ltd, assignee. A separating and culturing method of hair follicle stem cells. Korean patent KR2009021340A. 2009 Mar 3

109 **Park JK**, Yoon HH, Yoo BY, Kim YJ, Shin YH, inventors; Univ Dongguk Ind Academic Coop Found, assignee. Method for the preparation of dermal papilla tissue employing mesenchymal stem cells. WO2009014272A1. 2009 Jan 29

110 **Sherley James L**, King J, inventors; Massachusetts Inst Technology, assignee. Methods for ex vivo propagation of somatic hair follicle stem cells. WO2005121323A3. 2009 Apr 4

111 **Yoshimura K**, Inoue K, Sato T, inventors; Biomaster Inc, assignee. The cell transplant method for hair regeneration. Japanese patent JP2010082339A. 2010 Apr 15

112 **Lee L**, Chuong CM, Garner W, Jiang TX, inventors; Southern California University, assignee. Compositions and methods to generate pilosebaceous units. United States patent US20110321180A1. 2011 Dec 29

113 **Darling Thomas N**, Li S, Thangapazham R, inventors; Jackson H M Found Military Med, assignee. Hair follicle neogenesis. WO2011160055A2. 2011 Dec 22

114 **Armani A**, Armani S, Seneviratne C, Nazari R, inventors; Alvi Armani Genomics Inc, assignee. Cell Compositions and Methods for Hair Follicle Generation. United States patent US20110305671A1. 2011 Dec 15

115 **Lederman Seth M**, Kellogg Scott C, Ju W, inventors; Follica Inc, assignee. Combination therapy. WO2011123218A1. 2011 Oct 6

116 **Jin Y**, Zhang YJ, Yang L, inventors; Chinese PLA Fourth Military Medical University, Xi'an Engineering Technology Research Centre of Tissue Engineering, assignees. Tissue engineering skin with sebaceous gland-like structure and preparation method thereof. Chinese patent CN102172337A. 2011 Sep 7

117 **Huang YY**, Hsieh CH, Wang JL, inventors; Taiwan Nat Univ, assignee. Method and apparatus for culturing tissue. United States patent US20110293573A1. 2011 Dec 1

118 **Mize N**, inventor; Genogen Inc, assignee. Methods and compositions for skin regeneration. WO2011044367A1. 2011 Apr 14

119 **Naughton GK**, Ziegler F, Baumgartner M, Nickey K, inventors; Histogen Inc, assignee. Conditioned medium and extracellular matrix compositions from cells cultured under hypoxic conditions. WO2011006107A1. 2011 Jan 13

120 **Jin Y**, Fan XJ, Zhang YJ, Wang AJ, inventors; Shaanxi Ruisheng Biotechnology Co Ltd, Chinese Pla Fourth Military Medical Univ, assignees. Method for preparing tissue engineering skin containing hair follicle. WO2011094964A1. 2011 Aug 11

121 **Cotsarelis G**, Ito M, inventors; Pennsylvania Univ, assignee. Methods for generating new hair follicles, treating baldness and hair removal. Australian patent AU2012204059A1. 2012 Jul 26

122 **Mahjour SB**, Wang H, inventors; Mahjour, Seyed Babak, Wang, Hongjun, assignees. Creation of hair follicles in tissue-engineered skin grafts. United States patent US20120276154A1. 2012 Nov 1

123 **Sun G**, Zhang X, Harmon John W, Gerecht S, inventors; Johns Hopkins Univ, assignee. Skin and hair regeneration using polysaccharide-based hydrogels. WO2012158312A2. 2012 Nov 22

124 **Chen L**, Liu DQ, Lv Y, Pei XT, Shi W, Xie XY, Yao HL, Yue W, Xi JF, Zhang XY, inventors; Field Operation Blood Transfusion Institute of PLA Sciences Academy of Military, assignee. Cell culture medium and application thereof. Chinese patent CN102676448A. 2012 Sep 19

125 **Toyoshima KE**, Tsuji T, inventors; Organ Technologies Inc, assignee. Method of preparing regenerated hair follicle germ for transplantation in which hair color is controlled, composition including regenerated hair follicle germ for transplantation, and method of transplanting regenerated hair follicle germ. WO2012115079A1. 2012 Aug 30

126 **Yan QG**, inventor; Beijing Yonghe Hair Transplant Technolog, assignee. Culture method of hair follicle stem cell for treating baldness, as well as syringe. Chinese patent CN102586177A. 2012 Jul 18

127 **Toyoshima KE**, Tsuji T, inventors; Organ Technologies Inc, assignee. Method for producing regenerative organ primordium provided with guide for transplantation, composition containing regenerative organ primordium provided with guide for transplantation produced thereby, and method for transplanting regenerative organ. WO2012108069A1. 2012 Aug 16

128 **Liu J**, Li Y, inventors; Jilin Univ, assignee. Culture method for amplifying large numbers of hair follicle stem cells in vitro. WO2012009958A1. 2012 Jan 26

129 **Ishizaka S**, Oji Y, inventors; Nara Medical Univ, assignee. The cultivation method of a hair follicle stem cell. Japanese patent JP2012249556A. 2012 Dec 20

130 **Maya SB**, inventors; Newcastle-Upon-Tyne Univ, assignee. Expansion and directed differentiation of epidermal neural crest stem cells. WO2011144901A4. 2012 Mar 1

131 **Duan EK**, Zhang SB, inventors; Chinese Acad Sci Zoology Inst, assignee. A separating and culturing method of hair follicle stem cells. Chinese patent CN102344906A. 2012 Feb 8

132 **Lederman Seth M**, Kellogg Scott C, Barman Shikha P, inventors; Follica Inc, assignee. Hair growth treatment WO2012067630A1. 2012 May 24

133 **Yoshida Y**, Soma T, Fujiwara S, inventors; Shiseido Company Ltd, assignee. Composition for regenerating follicle which contains cd36-expressing connective tissue sheath cells.WO2012042618A1. 2012 Apr 5

134 **Yamanishi H**, Soma T, Yoshida Y, Ishimatsu Y, inventors; Shiseido Company Ltd, assignee. Method for promoting hair growth or hair regeneration by maintaining or increasing expression of cell-adhesion factor. United States patent US8334136B2. 2012 Dec 18

135 **Kurita K**, inventor; Lion Corp, assignee. Artificial skin. Japanese patent JP05097387B2. 2012 Dec 12

136 **Ehama R**, Kishimoto J, Ideta R, Soma T, inventors; Shiseido Company Ltd, assignee. Method of regenerating follicles by inhibiting gene capable of inhibiting follicle formation or activating gene capable of inducing follicle formation. Korean patent KR1186716B1. 2012 Sep 27

137 **Wan ACA**, Lim TC, Leong MF, Ying JY, Toh JKC, inventors; Agency Science Tech & Res, assignee. Fibrous structure. WO2013137829A1. 2013 Sep 19

138 **Liu HQ**, Guo XC, Wu MJ, inventors; PLA Second Military Medical University, assignee. Method of modifying mesenchymal stem cell by JAM1 gene and application thereof. CN103031278A. 2013 Apr 10

139 **Kobielak K**, Kandyba E, inventors; Southern California Univ, assignee. Hair follicle stem cells and methods of use same. WO2013040248A1. 2013 Mar 21

140 **Alenfall J,** Dunér P, Hultgårdh NA, inventors; Follicum AB, assignee. Novel compositions and uses thereof. WO2013021212A2. 2013 Feb 14

141 **Li Y**, Liu J, inventors; Jilin Univ, assignee. Culturing method for amplifying large amount of hair follicle stem cells in vitro. Taiwanese patent TW201321513A. 2013 Jun 1

142 **Gao Q**, Chen BL, Zeng Q, inventors; Gao Qing, Chen Bing-Lai, Zeng Qiao, assignee. Method for culturing amplified human hair follicle stem cells and reprogramming amplified human hair follicle stem cells to induced pluripotent stem cells. Chinese patent CN102994447A. 2013 Mar 27

143 **Fu XB**, Huang S, inventors; Chinese Pla No 1 Hospital Affiliated Gen, assignee. Construction method for tissue engineering skin containing complete skin appendage. Chinese patent CN102949752A. 2013 Mar 6

144 **Lin SJ**, Chan CC, Yen CM, inventors; Taiwan Nat Univ, assignee. Method for the manufacture of microtissues for inducing the growth of a hair follicle. United States patent US8492112B2. 2013 Jul 23

145 **Huang Z**, Lin X, Zhang YD, Chen SZ, inventors; Fujian Normal University, assignee. Method for differentiating human keratinized stem cell into human follicular cell through induction in vitro. Chinese patent CN103667180A. 2014 Mar 26

146 **Gho CG**, inventor; Hair Science Institute, assignee. Method for in vivo hair multiplication. WO2014092571A1. 2014 Jun 19

147 **Keyes W**, Doles J, inventors; Fundacion Privada Ct De Regulacio Genomica Crg, assignee. Jak inhibitors for activation of epidermal stem cell populations. WO2014013014A1. 2014 Jan 23

148 **Xu SC**, Quan RF, Huang ZM, Zheng X, inventors; Hangzhou City Xiaoshan Distr Traditional Chinese Medical Hospital, assignee. Separation culture method of rat hair follicle stem cells. Chinese

patent CN103710297A. 2014 Apr 9
149 **Aljitawi O**, Hopkins R, Detamore M, Garimella R, inventors; Childrens Mercy Hospital, Kansas Univ, assignees. Generating ck19-positive cells with hair-like structures from whartons jelly. United States patent US20140148915A1. 2014 May 29
150 **Christiano AM**, Jahoda CAB, inventors; Columbia New York Univ, assignee. The method for compact aggregation of a dermis cell. Japanese patent JP05386180B2. 2014 Jan 15

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REVIEW

# Structural properties of scaffolds: Crucial parameters towards stem cells differentiation

Laleh Ghasemi-Mobarakeh, Molamma P Prabhakaran, Lingling Tian, Elham Shamirzaei-Jeshvaghani, Leila Dehghani, Seeram Ramakrishna

Laleh Ghasemi-Mobarakeh, Elham Shamirzaei-Jeshvaghani, Department of Textile Engineering, Isfahan University of Technology, Isfahan, 84156-83111, Irans
Molamma P Prabhakaran, Lingling Tian, Seeram Ramakrishna, Department of Mechanical Engineering, Faculty of Engineering, 2 Engineering Drive 3, National University of Singapore, Singapore 117576, Singapore
Leila Dehghani, Department of Medical Science, Islamic Azad University, Najafabad Branch, Isfahan,81595158, Iran
Leila Dehghani, Isfahan Neurosciences Research Center, AlZahra Hospital, Isfahan University of Medical Sciences, Isfahan, 8174675731, Iran
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Correspondence to: Laleh Ghasemi-Mobarakeh, PhD, Department of Textile Engineering, Isfahan University of Technology, Daneshgah e Sanati HW, Isfahan 84156-83111, Iran. laleh.ghasemi@cc.iut.ac.ir
Telephone: +98-31-33911092
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## Abstract

Tissue engineering is a multidisciplinary field that applies the principles of engineering and life-sciences for regeneration of damaged tissues. Stem cells have attracted much interest in tissue engineering as a cell source due to their ability to proliferate in an undifferentiated state for prolonged time and capability of differentiating to different cell types after induction. Scaffolds play an important role in tissue engineering as a substrate that can mimic the native extracellular matrix and the properties of scaffolds have been shown to affect the cell behavior such as the cell attachment, proliferation and differentiation. Here, we focus on the recent reports that investigated the various aspects of scaffolds including the materials used for scaffold fabrication, surface modification of scaffolds, topography and mechanical properties of scaffolds towards stem cells differentiation effect. We will present a more detailed overview on the effect of mechanical properties of scaffolds on stem cells fate.

**Key words:** Stem cells; Tissue engineering; Differentiation; Mechanical properties; Surface modification; Topography

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**Core tip:** Application of stem cells in tissue engineering and regenerative medicine has attracted many researchers and it is considered as a hot topic of research. The research was mostly aimed towards the fabrication of a suitable scaffold with appropriate physical, chemical, biological and mechanical properties to guide the differentiation of stem cells to specific lineages of interest. This paper reviews the previous researches on effect of chemical structure, surface modification, topography and mechanical properties of scaffolds on stem cells behavior. Fabrication of scaffolds with desirable properties can open new strategies for cell therapy using tissue engineering technique.

Ghasemi-Mobarakeh L, Prabhakaran MP, Tian L, Shamirzaei-

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## INTRODUCTION

Autologous cells obtained from adult patients might deliver a less difficult route to regenerative-cell therapies. In the past, many tissues were assumed incapable of self-regeneration upon its damage, mainly because they do not possess any endogenous stem cells. However, recent discoveries show that more adult tissues harbor cells with capacity for regenerative repair[1]. Tissue engineering comprises of suitable cells, scaffolds and appropriate biochemical/physical factors, such that it function as a strategy towards the engineering of tissues and cellular delivery. Encapsulating stem cells within biocompatible scaffolds provide a matrix which result in cell adhesion, migration and differentiation suitable for transplantation and tissue regeneration[2-4].

Stem cells have uncovered a new perspective as therapeutic tools in regenerative medicine. These cells with the ability to proliferate indefinitely (*in vitro*) and the capacity to differentiate into any somatic cell type are a potential cell source. Stem cells are originated in the early embryo, the fetus, placenta, umbilical cord, and it occurs in many different tissues of the body. Also they have been engineered from somatic cells, termed as induced pluripotent stem cells (iPSCs). iPSCs give rise to different cell types including the neural cell types (neurons, oligodendrocytes, and glia), cardiomyocytes, osteoblasts, hepatocytes, and haemopoietic progenitors cells[5-8]. The two main stem cell types are the embryonic stem cells (ESCs) and the adult stem cells. ESCs are derived from fetal tissue or from the inner cell mass of the blastocytes with ability for unlimited growth in culture that could be associated to a high risk of teratoma formation. Several researches have been done on ESCs pluripotency to obtain specific cell lineages for tissue engineering and regenerative medicine, as well as for therapeutic treatments[8-13]. Adult stem cells are specialized cells including the mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and neural stem cells (NSCs)[9]. MSCs are self-replicating cells that are capable of differentiating in multidirectional pathways, resulting in cell lineages such as the osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, adipocytes, neural cells, oligodendrocytes and haemopoietic cells[9,14,15]. These cells have been used for the engineering of different tissues such as the cardiac[16] , tendon[17] , cartilage[18-20] , vascular[11,21] Meniscus[22], bone[23], Ligament[24], myocardia[25], fat[26], and neural tissues[5,16,17,27].

While a routine source of human MSCs is the bone marrow, they have been also derived from multiple adult tissues comprising of adipose tissue (ADSCs), umbilical cord blood, placenta, thymus, and dental pulp[28]. In comparison to MSCs derived from the bone marrow[29], ADSCs have several advantages such that they are easy to obtain after minor donor site morbidity, possess high proliferating capacity and can be preserved for longer period of time in cell banks[30]. Under appropriate culture conditions, ADSCs can differentiate to classical mesenchymal lineages, which include adipogenesis, chondrogenesis, osteogenesis, and myogenesis[31,32]. Moreover, the ability of ADSCs differentiation to numerous lineages when seeded into polymeric scaffolds has been well documented in several literatures[33,34].

The sources of HSCs are bone marrow, peripheral blood, umbilical cord blood, fetal hematopoietic system[35], ESCs and embryonic germ cells[11]. These cells are also a suitable cell source for blood vessel engineering[12]. Avoidance of anaesthesia without the need for hospitalization or blood transfusion, and low risk of serious adverse events are the major advantages of the peripheral stem cells, which make them the favourable source of stem cells worldwide. On the other hand, NSCs are defined as self-renewing multipotent progenitors existing in the developing and adult CNS. Generally, they are considered by their capacity to symmetrically self-renew and their ability to discriminate into neurons, oligodendrocytes, and astrocytes through asymmetrical fate-committed division[27]. Neural stem or progenitor cells can be obtained in different approaches such as direct isolation of these cells from embryonic or adult brain tissue[36,37]. NSCs have been used for tissue engineering of different tissues such as multiple cell types of the adult nervous system[5,38]. It was shown in several studies that stem cells behavior such as their adherence, proliferation and differentiation on scaffolds, depends on the material itself. Additionally, the surface chemistry, surface geometry/micro architecture of scaffolds, and the mechanical properties of scaffolds[39-42] play a significant role in determining the cellular responses after culture of stem cells on them[43].

Therefore, recent studies have focused on the selection of an appropriate biomaterial, whit focusing on the surface modification of the scaffolds, creating appropriate microstructures and controlling the mechanical properties of scaffolds to recapitulate the *in vivo* microenvironment suitable for regenerating tissues or for the differentiation stem cells to specific cell lineages[44].

This review will discuss on the influence of different aspects of scaffolds including scaffold composition, surface modification, micro-nano architecture of scaffolds and mechanical properties of scaffolds pertaining to stem cells differentiation. An emphasis is also given to the effect of mechanical properties of

scaffolds towards stem cells differentiation.

## EFFECT OF SCAFFOLD COMPOSITION ON STEM CELLS DIFFERENTIATION

The interaction of stem cells with their surrounding microenvironment is fundamental to multiple processes such as cell migration, proliferation, lineage specificity, and tissue morphogenesis[45]. Biomaterials play an important role in directing tissue growth and chemical properties of the scaffolds have been shown to influence the behavior of stem cells whereas the scaffold composition has an significant role in stem cells differentiation towards preferred lineages[42,43,45]. The ability to selectively guide stem cells differentiation by merely changing the properties of an underlying biomaterial scaffold is a smart approach in tissue engineering, which can help compliment or potentially eliminate the use of exogenous differentiation inducers like the viral gene vectors and small molecule drugs[46].

Natural polymers such as the collagen, fibrinogen, hyaluronic acid, glycosaminoglycans, cellulose, chitosan, silk fibroin, *etc.*, consist of components found in the native extracellular matrix, such that they can assist cells to attach to cell surface receptors and provide a physical environment to control cell function compared to synthetic materials[40,47]. However these materials suffer from fast degradation rates, difficulty in sterilization and purification, high variability, high contamination potential, and induce immune response upon implantation, while synthetic materials offer the potential for improved control, repeatability and safety[40,47]. Scaffolds fabricated by synthetic materials can be chemically modified by natural materials to carefully regulate the differentiation of stem cells[47]. Immune response is an important concern in synthetic biomaterials used for scaffold fabrication and it can be reduced by selection of materials which are inert inherently or by modification of scaffolds to avoid detection by the immune system. Hydrophobic materials usually tend to increase monocyte adhesion compared to hydrophilic materials leading to a local immune reaction at the scaffold site[48].

Awad *et al*[34] compared the chondrogenic differentiation of human adipose-derived adult stem cells seeded in alginate, agarose and gelatin scaffolds and their findings revealed that the growth and differentiation potential of adult stem cells seeded on the scaffolds varied and the cells on gelatin scaffolds showed better proliferation and differentiation. Collagen has attracted much interest as a biomaterial for fabrication of scaffolds in tissue engineering as a key structural protein found in the extracellular matrix of many connective tissues[49,50]. In spite of the several advantages of collagen as a biodegradable, biocompatible, safe and multifunctional material, its potential to evoke immune response has raised concerns as an animal-derived biomaterial[51].

Meinel *et al*[52] investigated the osteogenic differentiation of human MSCs on different protein substrates including unmodified collagen with fast degradation rate, cross-linked collagen with slow degradation rate and silk; in both static and dynamic culture conditions. Under dynamic condition, MSCs on cross-linked collagen and silk scaffolds deposited more calcium and had a higher alkaline phosphatase activity than MSCs on unmodified collagen scaffolds indicating the importance of scaffold properties in causing osteogenesis of the cultured MSCs[52].

Jäger *et al*[39] cultivated MSCs on the D,D,L,L-polylactide (PLLA), collagen I/III, and polygalactin-910/polydioxanone (PGPD) scaffolds and their results showed significant differences in the proliferation, differentiation, and $Ca^{2+}$ accumulation of MSCs on different scaffolds. Cell adhesion on PGPD was lower compared to the cell adhesion on PLLA and collagen I/III scaffolds, which was related to the significant biodegradation rate of PGPD scaffolds compared to other scaffolds. They found significantly higher cell numbers on collagen I/III membrane compared to that for PLLA scaffolds after 4 h cell culture while the differences were less significant after 28 d of cell seeding. Lower cell number on PLLA scaffolds compared to collagen I/III after 4 h could be attributed to the hydrophobic nature of PLLA while an increasing deposition of serum proteins from culture media on the PLLA scaffold may reduce the effect of its hydrophobic nature and decreased the quantitative differences in cell attachment between collagen I/III and PLLA scaffolds after 28 d cell seeding[39].

PEG is a relatively inert ,biocompatible and hydrophilic polymer[53]. Hwang *et al*[41] encapsulated MSCs in PEG based hydrogels containing type I collagen, type II collagen and hyaluronic acids (HA) which are the main components of musculoskeletal tissue matrix. Chondrogenic differentiation of MSCs was slightly enhanced in collagen-matrix-based hydrogels, whereas osteogenic differentiation was induced in HA-containing hydrogels suggesting the potential of ECM components to modulate the fate of MSCs differentiation[41].

Ravichandran *et al*[54] fabricated a composite scaffold containing PLLA and Poly-benzyl-L-glutamate (PBLG), a polymer of glutamic acid in which the carboxyl groups have been benzoylated, by electrospinning. Nanohydroxyapatite (n-HA) was also deposited on the surface of the nanofibers by calcium-phosphate dipping method. Their results proved that the incorporation of PBLG along with n-HA deposition on the surface of scaffolds promoted greater osteogenic differentiation of ADSCs in the absence of an induction medium highlighting the chemical composition of the scaffold as a determinant factor towards ADSCs differentiation[54].

Shah *et al*[46] reported selective differentiation of NSCs into oligodendrocytes by seeding NSCs in PCL nanofibrous scaffolds coated by graphene oxide (GO).

Higher concentration of GO was found to promote NSCs differentiation into mature oligodendrocytes without introducing differentiation inducers in the culture media. Their results showed that the presence of GO in the structure of nanofibrous scaffolds was a crucial factor that determined the stem cell-scaffold interactions in directing NSCs towards oligodendrocyte differentiation[46].

One of the major challenges in cartilage and disc-tissue engineering using MSCs is the rapid expression of type Ⅹ collagen as a marker of chondrocyte hypertrophy associated with endochondral ossification. Nelea *et al*[55] used polypropylene and nylon-6 polymers for the fabrication of scaffolds and modified the surface of scaffolds by glow discharge plasma treatment in ammonia gas to investigate the potential of each polymer in its abilityto inhibit the expression of type Ⅹ collagen. They cultured MSCs on modified and un-modified polypropylene and nylon-6 and evaluated the chondrogenic differentiation pathway using aggrecan and types Ⅰ, Ⅱ, and Ⅹ collagens. Their results showed that MSCs did not adhere to unmodified PP while they observed cell adhesion on modified PP, nylon 6 and unmodified nylon 6 and concluded that the nature of the surface should be vitalfor the interaction of MSCs. Both modified and un-modified nylon-6 suppressed the expression of type Ⅹ collagen, while the modified polypropylene scaffold almost completely inhibited its expression indicating the impact of polymer type and surface characteristics on the fate of stem cells[55].

Hosseinkhani *et al*[56] encapsulated DNA nanoparticles into collagen sponge reinforced with poly (glycolic acid) fiber for bone tissue engineering. More osteogenic differentiation of MSCs and formation of homogeneous bone upon implantation of scaffolds containing DNA nanoparticles was observed by these researchers[56].

In yet in another study, semi-interpenetrating polymer networks made of collagen type Ⅰ fibers combined with fibronectin or laminin were fabricated to investigate the effect of matrix composition towards ESCs differentiation. Results showed that scaffold composition has an important role in ESCs development and differentiation whereas the presence of fibronectin in collagen scaffolds strongly induces endothelial cell differentiation and vascularization. On the contrary, laminin stimulated ESCs to differentiate into beating cardiomyocytes[57]. Costa-Pinto *et al*[58] prepared chitosan-poly (butylene succinate) scaffolds in different weight ratios of chitosan (50%, 25%, and 0%) and compared the potential of osteogenic differentiation of human bone marrow stromal cells on these scaffolds. Their results revealed higher cell viability, adhesion, proliferation, and osteogenic differentiation on scaffolds with more ratios of chitosan[58]. Overall, it was clear that the scaffold material and its properties played a critical role in directing the stem cells towards differentiation, with proteins and peptides had a positive influence towards this effect.

## EFFECT OF SURFACE MODIFICATION OF SCAFFOLDS ON STEM CELLS DIFFERENTIATION

Cell-biomaterial interactions has a significant role on the growth and differentiation of stem cells within scaffolds[34]. It is well known that the surface properties of scaffolds have an important role in stem cells-biomaterials interactions, ultimately controlling the stem cell adhesion, proliferation, and differentiation after its attachment on the surface[59,60]. Stem cells differentiation can be regulated by presenting suitable biological or chemical signals within the structure of the scaffolds[47]. Therefore the chemical and biological modification of the scaffolds can affect stem cells behavior which is also defined by the substrate properties and their degradation rate, eventually manipulating the signal transduction pathways in stem cells[47]. It can be concluded that the surface chemistry of materials can affect protein adsorption and the binding of different integrins and influence the cell behavior[59]. Surface modification of scaffolds is therefore an important issue in tissue engineering in order to control the cellular behavior[59]. More recently different methods have been established for the modification of scaffolds thorough incorporation of biomolecules within the scaffold structure by method like simple physical adsorption or by covalent conjugation[47,61].

Ayala *et al*[45] demonstrated the influence of interfacial matrix hydrophobicity on stem cells behavior. They fabricated tunable synthetic scaffolds with control over the hydrophobicity of the scaffolds, by copolymerizing acrylamide with acryloyl amino acids and their results showed that the small changes in matrix hydrophobicity can dramatically alter the cell-matrix interaction and can influence various cellular behaviors[45].

Chua *et al*[62] investigated the effect of surface-functionalized polyethersulfone (PES) scaffolds including the hydroxylated, carboxylated and aminated PES nanofibers and films and their results showed that the surface-bound amino groups highly influenced the proliferation and differentiation of hematopoietic stem/progenitor cells. The effect of coating of polycaprolactone-co-lactide scaffolds with collagen Ⅰ (coll Ⅰ) and coll Ⅰ/chondroitin sulfate (CS) on osteogenic differentiation potential of ovine bone marrow MSCs has been investigated. The results showed surface modification of scaffolds using coll Ⅰ/CS induce osteogenic differentiation of cells, without using any differentiation supplements such as dexamethasone, revealing the osteoinductive characteristics of the modified scaffolds[63].

Rim *et al*[64] fabricated electrospun PLLA nanofibrous scaffolds and coated scaffolds with polydopamine through immersion in an aqueous solution of dopamine with mild shaking for 1 h. Their results showed more

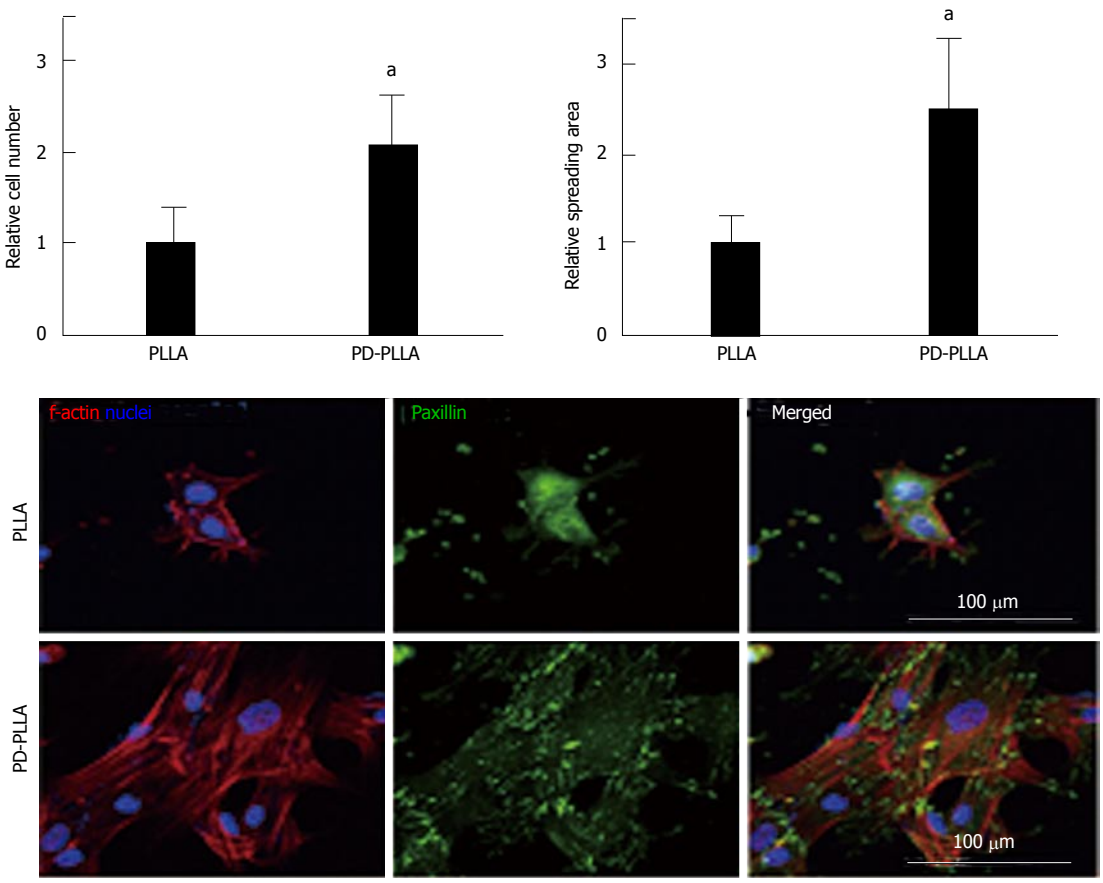


**Figure 1 Quantitative analysis of initial cell adhesion from human mesenchymal stem cells cultured on the fibers.** Relative adherent cell numbers and spreading area of human mesenchymal stem cells (hMSCs) cultured on PLLA and PD-PLLA fibers were analyzed after 12 h of culture. $^{a}P < 0.05$, PD-PLLA *vs* PLLA group. Adherent morphology of hMSCs on PLLA and PD-PLLA fibers was observed by confocal microscopy. Scale bars represent 100 μm. Reproduced with permission from Rim *et al*[64]. PLLA: Poly(l-lactide); PD-PLLA: Poly(l-lactide) (PLLA) fibers coated with polydopamine.

adhesion and proliferation of MSCs cultured on surface modified nanofibrous scaffolds with dopamine relative to cells seeded on unmodified scaffolds (Figure 1). More osteogenic differentiation as well as angiogenesis was also observed for MSCs seeded on modified scaffolds compared to unmodified scaffolds[64].

In yet another study, electrospun PCL nanofibers were coated using polydopamine by simple immersion of substrates in an alkaline dopamine solution. RE-1 silencing transcription factor (REST) was then absorbed onto PCL nanofibers coated with polydopamine to induce scaffold-mediated gene knockdown for enhanced neuronal differentiation of neural stem/progenitor cells. The results showed significant enhanced neuronal commitment and decreased glial cells differentiation due to presence of the silencing of REST[44]. Controlled release of insulin-like growth factor Ⅰ (IGF-I) from silk fibroin scaffolds for chondrogenic differentiation of human MSCs has been studied by Uebersax *et al*[65] and the results showed that IGF-Ⅰ loaded silk fibroin scaffolds have the potential to provide chondrogenic stimuli to human MSCs, thus beneficial for cartilage repair.

In yet another study, copolyester of 3-hydroxybutyrate-cohydroxyhexanoate (PHBHHx) scaffolds were fabricated and coated with PHA granule-associated proteins (PhaP) and PHA granule-associated proteins PhaP fused with RGD peptide (PhaP-RGD) for investigating the effect of surface modification towards chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs). Their results showed that the surface modification of scaffolds with PhaP-RGD promote chondrogenic differentiation of hBMSCs compared to PhaP coated or uncoated scaffolds even without the presence ofchondrogenic induction medium (Figure 2). Hyaline cartilage regeneration, and inhibited fibrocartilage formation in hBMSCs derived chondrocytes was also observed on PhaP-RGD coated PHBHHx scaffolds indicating suitability of this substrate for cartilage tissue engineering compared to the uncoated ones[61].

In yet another study, poly (ethylene glycol) (PEG) hydrogels were functionalized with heparin and osteogenic differentiation of human MSCs was evaluated. The results through increased ALP production and gene expression of osteopontin and collagen type Ⅰ showed that functionalization of hydrogel with heparin induce osteogenic differentiation which is likely due to improvement of cell-scaffold interactions due to the presence of heparin[66]. Surface modification of poly-(lactic-*co*-glycolic acid) (PLGA) (75:25) scaffolds with RGD peptides also enhanced the human

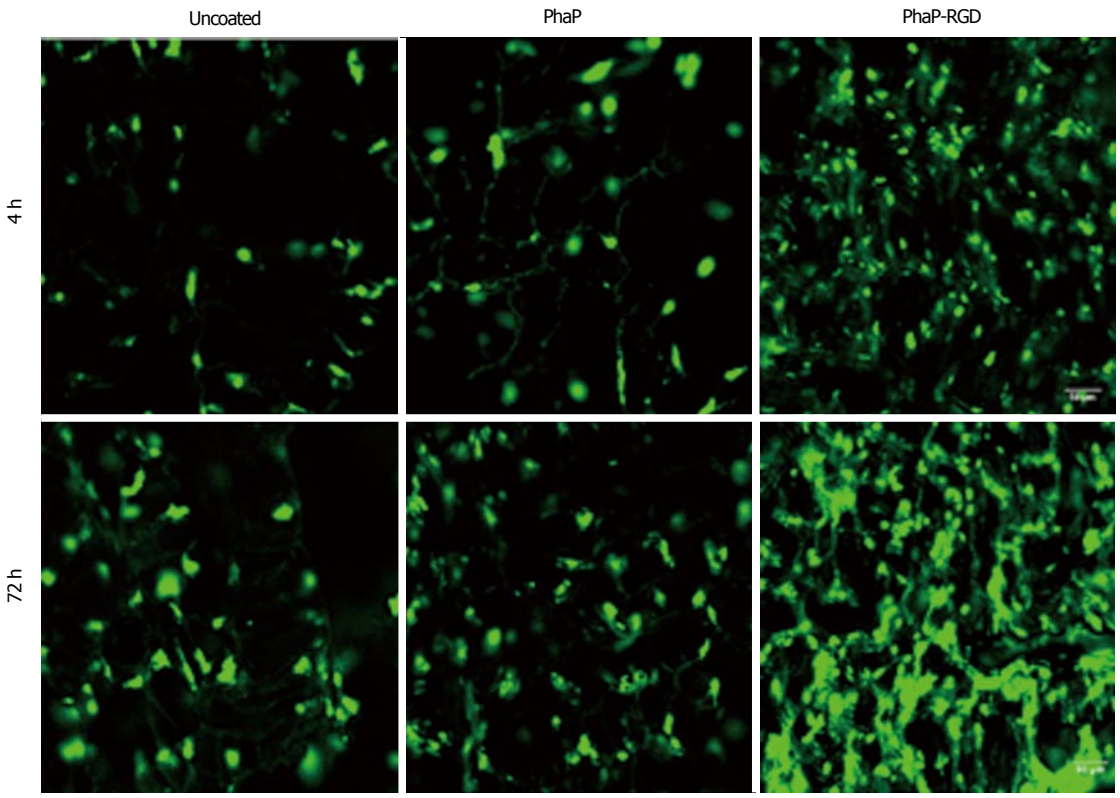


**Figure 2 Confocal microscopic imaging of human bone marrow mesenchymal stem cells grown in uncoated, PhaP and PhaP-RGD coated 3-hydroxybutyrate-cohydroxyhexanoate scaffolds after 4 or 72 h of incubation, respectively.** Phalloidin-fluorescein isothiocyanate was used to F-actin of cells grown in the scaffolds (Green). Reproduced with permission from You *et al*[61]. PhaP-RGD: PhaP binding protein fused with arginyl-glycyl-aspartic acid.

osteoprogenitor differentiation and osteogenesis[67]. While RGD peptide immobilized macroporous alginate scaffolds showed enhanced chondrogenesis properties of human MSCs compared to the un-modified ones[68].

*In vitro* osteogenic differentiation of human MSCs was also observed on RGD peptide functionalized PLLA nanofibersby Paletta *et al*[69]. Their results showed more osteogenic differentiation of human MSCs on modified scaffolds revealing the osteoinductive effect of the scaffolds functionalized with RGD[69].

Kuo *et al*[70] fabricated PLGA /chitosan scaffolds and functionalized it with type I collagen, whereby these researchers were able to improve the cell adhesion and viability on PLGA/chitosan/collagen scaffolds. Moreover, MSCs differentiated towards osteoblasts in the modified scaffolds without induction procedures, while neural differentiation was observed on the scaffolds by the induction MSCs with neuron growth factor (NGF)[70].

In another study Yang *et al*[71] fabricated porous poly l-lactide-co-ε-caprolactone (PLCL) and did surface modification *via* crosslinking of chitosan on the surface of scaffold. Their finding showed elongated morphology of MSCs on modified scaffolds while cells on unmodified scaffolds showed more spherical morphology with lower spreading. Moreover, the surface modified scaffolds provide surfaces for early differentiation of MSCs with more *in vitro* cartilage tissue formation revealing more condrogenic differentiation of MSCs on modified scaffolds compared to the unmodified PLCL scaffold[71].

In yet another study, Budiraharjo *et al*[72] obtained carboxymethyl chitosan scaffolds (CMCS), coated with hydroxyapatite and compared the behavior of osteoblasts and human MSCs on both modified and un-modified scaffolds. Coating the scaffold with HA substantially enhanced the osteogenic differentiation of the human MSCs.

Surface modification using plasma has been frequently used to improve surface properties of scaffolds fabricated with synthetic polymeric materials through formation of functional groups on the surface[73]. Lin *et al*[74] investigated the effect of modification of PLLA scaffolds by gas plasma towards the differentiation of ADSCs and their results showed that the cells seeded on modified scaffolds displayed significantly increased differentiation into endothelial cells[74].

Wang *et al*[60] fabricated porous nanocrystalline HA/chitosan scaffolds using a lyophilization technique, further treated them with cold atmospheric plasma as a simple, quick, and inexpensive method to modify the surface of scaffolds. Their results showed enhanced stem cell growth and osteogenic differentiation on modified scaffolds, which might be due to higher amounts of protein adsorption on the scaffold surfaces due to improves surface roughness and wettability after its modification[60].

An ideal bone implant should recruit osteoblasts or

progenitor cells from the adjacent native tissue and induce cell proliferation and osteogenic differentiation for new bone formation. Hu *et al*[75] modified Ti6Al4V (TC4) implants using biofunctional multilayer coating of gelatin, chitosan, gelatin and human recombinant BMP2. *In vitro* results showed that such modifications of the implant surface stimulated the adhesion and osteogenic differentiation of MSCs and *in vivo* results showed improved induction of new bone formation at the interface of bone and implant and the integration of the implant within the surrounding living tissues[75] .

## EFFECT OF SCAFFOLD TOPOGRAPHY ON STEM CELLS DIFFERENTIATION

The topography of the extracellular matrix is known to influence cell attachment, morphology, proliferation and differentiation[76]. Topography can also enhance differentiation of progenitor cells into their programmed pathways[77].

Topographic surfaces with micro and nano scale structures have been shown to stimulate the cell alignment, polarization, elongation, migration, proliferation, gene expression, *etc.*, while the mammalian cells have been shown to react to nanoscale structures on a synthetic surface[77,78]. Nano scale topographies are obtained by different fabrication methods such as electrospinning, polymer phase separation, photolithography, chemical vapor deposition, electron beam lithography, *etc.*[2]. The similarity of electrospun nanofibers to the morphology of native extracellular matrix has attracted interest in the application of these scaffolds as a substrate for stem cell attachment and differentiation[2].

Smith *et al*[79] compared the in *vitro* osteogenic differentiation of ESCs on nanofibrous scaffolds and traditional scaffolds with two and three dimensional structure without the nanofibrous features. The results showed that the nanofibrous features enhanced the osteogenic differentiation and mineralization of ESCs compared to traditional substrates. Moreover, the osteogenic differentiation was observed on nanofibrous scaffolds without osteogenic supplements, while cells on other scaffolds required osteogenic supplements and growth factors for osteogenic differentiation[79].

Ragetly *et al*[80] evaluated the chondrogenic differentiation of MSCs on chitosan microfibrous scaffolds and chitosan sponges. More chondrogenesis differentiation was observed on chitosan microfibers compared to that on sponges indicating the effect of scaffold microstructure towards the cell behavior[80].

Christopherson *et al*[81] evaluated the effect of topographical features of nanofibrous scaffolds in view point of fiber diameter on rat adult neural stem/progenitor cell (rNSC) differentiation and proliferation. They fabricated different nanofibrous scaffolds with average fiber diameters of 283 ± 45 nm, 749 ± 153 nm and 1452 ± 312 nm and their findings showed that the fiber diameter of PES nanofibrous scaffolds significantly influenced the differentiation of rNSCs, whereas by decreasing of fiber diameter, more cell spreading and proliferation was observed. Additionally, the differentiation of stem cells towards oligodendrocytes was observed on nanofibrous scaffolds with fiber diameter of 283 ± 45 nm while morphology similar to neuronal progenitors was observed on nanofibers with fiber diameter of 749 ± 153 nm. Although, the number of cells on nanofibers with fiber diameter of 1452 ± 312 nm was found to be low which may be due to the difficulty of adhesion and migration of rNSCs on fibers with large diameter. However, the cells present within these nanofibrous scaffolds showed the morphology of neurons or neuronal progenitors[81].

In yet another study, aligned PLLA nanofibrous scaffolds with a mean diameter ranging from 307 to 917 nm and random PLLA nanofibrous scaffolds with a mean diameter ranging from 327 to 1150 nm were fabricated and the behavior of NSCs were evaluated on these scaffolds. The results showed that the depending on the fiber diameter and pattern, NSCs behaved differently revealing that the fiber alignment and diameter had significant effects on cellular behavior[82].

Yin *et al*[76] examined the effect of alignment of PLLA nanofibrous scaffolds on differentiation of stem cells from human tendon andthe differentiation of the cells seeded on aligned nanofibrous scaffolds was found to be higher with either normal or osteogenic media than on the randomly-oriented nanofibers[76].

Subramony *et al*[83] also evaluated the effect of PLGA nanofiber alignment and mechanical stimulation towards MSCs differentiation. Their results showed fibroblastic differentiation of MSCs in the absence of chemical induction factors on aligned nanofibers together with mechanical stimulation under tensile loading. No fibroblastic differentiation was observed on the non-aligned scaffolds even after the application of mechanical stimulation, indicating the effect of topography of scaffold towards stem cells differentiation[83].

Bakeine *et al*[84] investigated the effect of nanoscale surface topography on adhesion, proliferation and neural differentiation of mouse ESCs by fabrication of thin films of gold with different root mean square (RMS) surface roughness of 10, 21, 30 nm and 50 nm. Their results showed that surface topography influencedthe neural differentiation whereas highest differentiation was observed on gold films with RMS surface roughness of 21 nm after five days of cell seeding without addition of the traditional soluble neurotrophic factors[84].

The effect of topography with different sizes (groove width: 350 nm/2 μm/5 μm, depth = 300 nm) on the differentiation of human induced pluripotent stem cells (hiPSCs) towards neuronal lineage was studied by Pan *et al*[78]. Their results showed the obvious effect of topography on directing differentiation of hiPSCs towards neuronal lineage with noticeable up-regulation of neuronal marker expression on surfaces with nanostructured topography[78].

Yim *et al*[77] studied the proliferation and diffe-

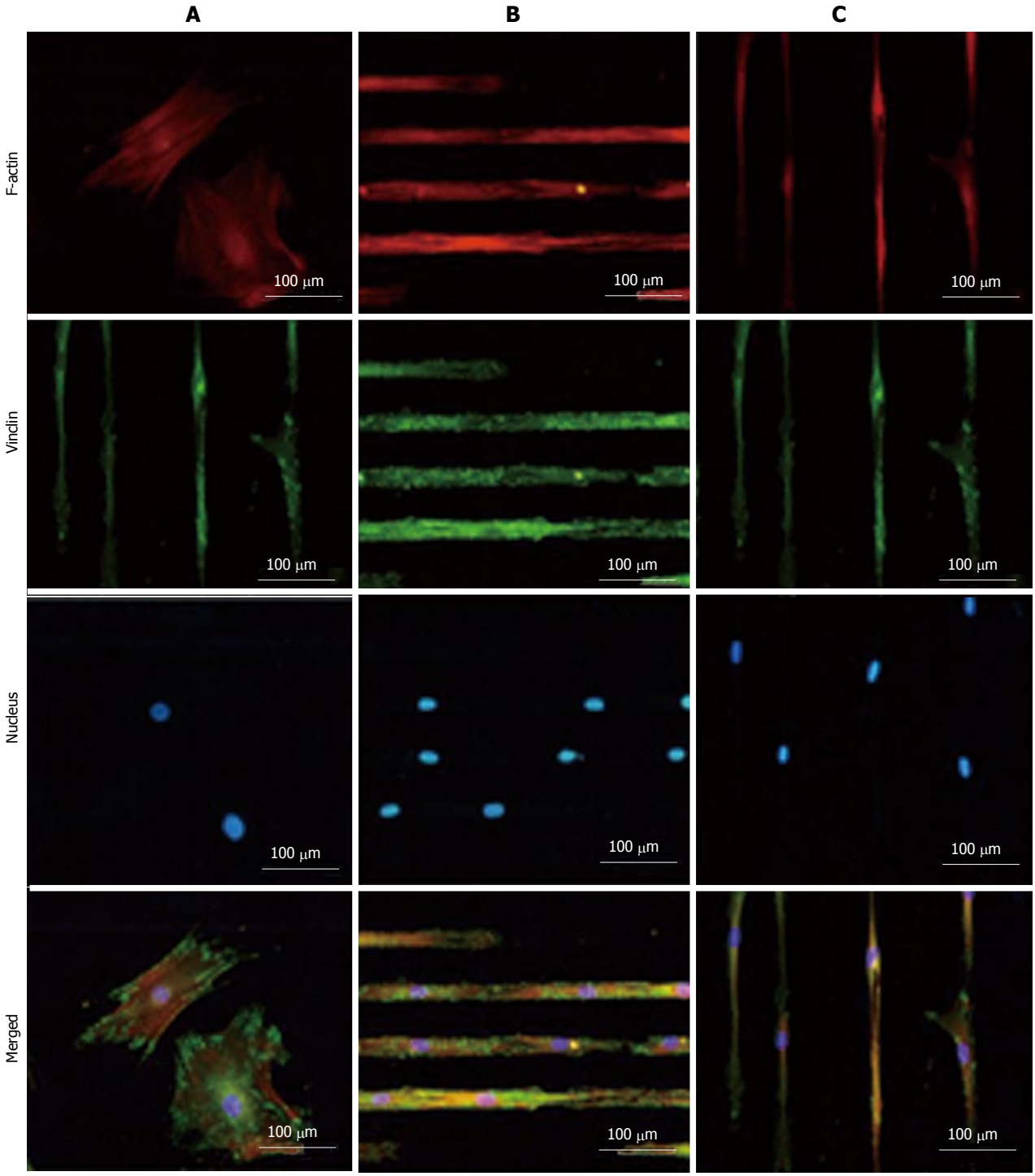


**Figure 3 TRITC-Phalloidin labeled F-actin (red), AlexaFluor 488 labeled vinculin (green), diamidino-2-phenylindole nuclear staining (blue) and overlaid fluorescent image of immuno-stained cellular components (merged) for the unpatterned (A) and patterned human mesenchymal stem cells (B and C).** Samples were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution for 4 d before they were fixed and stained. All images were taken with a 20 × objective lens. (Scale bar = 100 μm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Reproduced with permission from Tay *et al*[86].

rentiation of human MSCs on nano-gratings of 1 μm, 10 μm, 350 nm width and their results showed the alignment of cytoskeleton and nuclei of human MSCs along the nano-gratings. Significant up-regulation of neuronal markers was also observed on patterned surface compared to un-patterned and micro-patterned controls[77].

Chaubey *et al*[85] evaluated the effect of the surface topography on the differentiation of multipotent mouse bone marrow stromal precursors (D1 cells) to adipocytes. They compared the differentiation potential of D1 cells on patterned PLLA films, plain PLLA films and tissue culture-plate and their results showed the impact of patterned surfaces on cell differentiation[85].

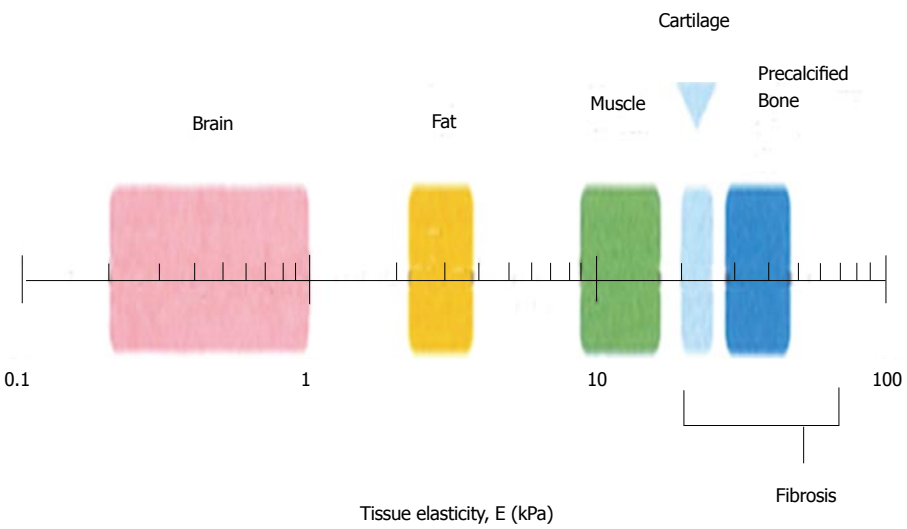


**Figure 4 Schematic depicting the normal variation in elasticity of the indicated tissue[92].**

Tay *et al*[86] printed fibronectin on PLGA thin films and investigated the differentiation of human MSCs on patterned and un-patterned scaffolds, where they revealed the differentiation of human mesenchymal stem cells (hMSCs) into myocyte like cells on the micropatterned films (Figure 3).

Shi *et al*[87] fabricated two nano-grating substrates with a period of 250 and 500 nm and with a depth of 120 nm on quartz, and investigated the effect of substrate nanotopography on differentiation of ADSCs into endothelial cells which play an important role in vascularization. Decreased cell proliferation along with enhanced endothelial gene expression of the ADSCs and *in vitro* angiogenesis was observed on nanograting ssuggested the role of nanotopography on ADSCs differentiation to endothelial cells[87]. Oh *et al*[88] fabricated PCL cylindrical scaffolds with different pore size (90-400 μm range) along the longitudinal direction; and investigated the effect of pore size on the chondrogenic differentiation of ADSCs. Their results showed that the pore size of 370-400 μm provided a more suitable environment for chondrogenic differentiation than other pore size groups[88].

Teo *et al*[89] applied nanotopography technique along with NGF controlled release from polydimethylsiloxane substrate for neuronal tissue engineering and their results showed enhanced expression of neuronal genes on nanopatterned substrate combined with NGF delivery[89].

The influence on nanotopography, whether in the form of fibers, porous microstructures or pits and grates, was studied extensively by various researchers. However, comparison of the results between such studies was difficult due to the differences in various factors involved in each study, where some researchers used mechanical stimulation, while others used a specific biomolecule for the differentiation to take place. A systematic evaluation is therefore necessary, to clearly define the fiber length or the pore size that might specifically cause the differentiation of stem cells to nerve, osteolineages or endothelial cells.

## EFFECT OF CULTURE ENVIRONMENT ON STEM CELLS DIFFERENTIATION

Bioreactors have been used in tissue engineering to overcome the drawbacks of static culturing conditions and to obtain uniform distribution of cells within the scaffolds and provide sufficient levels of oxygen, nutrients, cytokines, and growth factors, and to expose the cultured cells to mechanical stimuli[90]. Dynamic condition of cell culture has also been shown to influence stem cells differentiation.

Gomes *et al*[90] evaluated the effect of static and dynamic culturing conditions on the proliferation and osteogenic differentiation of rat bone marrow stromal cells seeded on two starch-based scaffolds. Calcium content analysis, VonKossa-stained sections, tetracycline fluorescence and histological analysis revealed the enhancement of osteogenic differentiation under dynamic condition created by flow perfusion bioreactor[90]. Mauney *et al*[91] also showed increased levels of alkaline phosphatase activity, bone-specific protein transcript levels, and mineralized matrix production by human bone marrow stromal cells under dynamic culture condition indicating increase inosteogenic differentiation of human BMSCs using mechanical stimulation applied by bioreactor. However, the influence of mechanical stimulation towards stem cells differentiation is not studied highly and more investigations are required.

## EFFECT MATRIX MECHANICAL PROPERTIES TOWARDS STEM CELL DIFFERENTIATION

Matrix stiffness varies with respect to different organs

present in the human body, and Figure 4 shows the difference in matrix stiffness of a few organs, as depicted by Vincent *et al*[92]. It explains how the matrix stiffness varies throughout the body: the brain is the softest tissue with an elasticity ranging between 0.2 to 1 kPa, while the precalcified bone is the hardest tissue with an elasticity value of more than 30 kPa[87], explaining the differences in the tissue matrix stiffness. Cells of different lineages survive in distinct natural environment, and they possess different stiffness. This is also the main reason why we believe that the stiffness have a higher influence towards the differentiation of stem cells. For tissue engineering, the most considered factors are the structure and the components used for scaffold fabrication, which determine the physical, chemical and biological properties of the scaffolds, and these properties will furthermore affect the differentiationof stem cells.

### Bone

Various strategies has been acknowledged and selectively chosen to alter the biomaterial surfaces or to create micro-environment to establish both mechanical and biochemical cues for the differentiation of stem cells, especially for application in regenerative medicine. Chemical factors have been individually studied, suggesting the ability of BMP-2 to promote pro-osteogenesis or IGF to promote tenogenic phenotype[93,94]. However, aligning the mechanical properties of the scaffolds with the specific tissue of interest was an interesting strategy applied to hydrogels, nanofibers and ceramics, in order to advance the field of stem cell differentiation on bioengineered substrates. Among these, injectable biodegradable hydrogels eliminate the need for surgical interventions or they allow for minimally invasive procedures causing minimal harm to the patients. However, physically crosslinked hydrogels lack strength and stability, while the chemically crosslinked hydrogels are more appreciated due to their high mechanical strength and stiffness. Hydrogels are useful substrates that assist to determine the interplay between physical cues/stiffness and their relationship with the fate of stem cell differentiation. A thiolene chemistry was utilized by Gandavarapu *et al*[95] to functionalize poly(ethylene glycol) hydrogels with a pendant peptide moiety, c(RRETAWA) and gels with Young's Modulus (YM) of 2 kPa (soft) and 25 kPa (Stiff) were prepared. The stiffer substrate was found to give 3.5 fold higher hMSCs attachment. The ALP activity of hMSCs cultured on stiff gels containing 0.1 mmol/L and 1 mmol/L c(RRETAWA) increased by 2.5 and 3.5 fold, respectively after 14 d of cell culture. At the same time, the expression of osteopontin and collagen-1a for cells cultured on stiffer gels were higher, suggesting the application of high substrate stiffness gels for osteogenic differentiation of MSCs. In order to adjust the matrix stiffness, crosslinking of the thiol functionalized hyaluronic acid (HA-SH) and thiol functionalized recombinant human gelatin (Gtn-SH), were performed by using varied amounts of poly (ethylene glycol) tetra-acrylate (PEGTA)[96]. Further hMSCs were seeded on the hydrogels to assess their potential towards osteo-lineage differentiation. MSCs grown on gels with 1.5 kPa stiffness, was found to express the early bone protein, namely osteopontin, while the cells on stiffer (4 kPa) gel expressed late osteogenic gene or the bone sialoprotein (BSP). This highlights the potential of higher osteogenic differentiation on stiffer hydrogels, mainly because the focal adhesion structures might have increased and F-actin might have got organized on such substrates, supporting the differentiation of MSCs to osteo lineages.

In order to study the role of substrate stiffness towards cell differentiation, Sun *et al*[97] prepared crosslinked gelatin scaffolds, where by the influence of scaffold strength was demonstrated *in vitro* and *in vivo* during this study. Crosslinking of gelatin scaffolds was performed by these researchers, using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) to prepare three dimension gelatin scaffolds with high stiffness properties. The elastic modulus of the EDC crosslinked scaffolds (2.5 kP) was significantly higher than those of the non-crosslinked scaffolds (0.6 kP). High mechanical strength of cross-linked scaffolds was suggested to promote stem cell mediated bone regeneration *via* endochondral ossification process, than the non-crosslinked scaffolds. *In vivo* studies using Inbred C57BJL/6 mice, showed significantly high trabecular bone formation for the crosslinked gelatin implanted animals, by micro-computed tomography and histology analysis[92]. Wang *et al*[98] prepared gelatin-hydroxyphenylpropionic acid-tyramine (Gtn-HPA-Tyr) conjugates to simulate osteogenic differentiation of hMSCs, by conjugation of Tyr to Gtn-HPA conjugate by means of carbodiimide/active ester mediated coupling reaction. Oxidative coupling of phenol moieties were further carried out by hydrogen peroxide ($H_2O_2$) and horseradish peroxidase (HRP), whereby the concentration of $H_2O_2$ was varied producing Gtn-HPA-Tyr hydrogels of varying storage modulus (G0). Hydrogels with G0 > 20000 was found suitable for the osteo-specific differentiation of MSCs, with osteocalcinupregulation and high calcium accumulation was observed on these gels compared to hydrogels with G0 of 13500. Stiffer hydrogels were suggested beneficial for the repair of bone defects. In yet another attempt, the ability of cells to differentiate to osteogenic cells were studied on transglutaminase cross-linked gelatin (TG-Gel) of varying rigidity and these researchers found that thesofter matrix promote cell proliferation, while stiffer matrix promote osteogenesis[99]. Osteogenic differentiation was found to occur in 6% and 9% TG-Gels with rigidity of 13.51-2.13 kPa and 32.32-1.9 kPa, respectively than using 3% TG-Gels. However, little or no effect of BMP-2 was found in the case of stiffer (6% and 9%) TG-Gelstowards MSC differentiation. The 9% TG-Gels with and without BMP-2 demonstrated high

calcium deposition with expression of osteogenic markers during *in vitro* studies. On the other hand, the ALP activity and early markers of osteogenic differentiation were observed in 6% TG-Gel and these cells differentiated to osteoblast-like cells at a later time point. Stiffer substrates are also demonstrated to direct the osteogenic differentiation of ADSCs, even in the absence of any growth factors[100]. PDGF and BMP-2 were covalently immobilized to collagen-Glycosoaminoglycan (CG) membrane in defined patterns, by combined photolithography-carbodiimide crosslinking strategy and the elastic modulus (stiffness) of the matrix was orthogonally manipulated. These researchers explained that by increasing the ratios of EDC:NHS:COOH, the stiffness of the CG got increased. Photoimmobilized BMP-2 was demonstrated to have a little effect on lineage-specific gene expression of ADSCs during this study, while the matrix stiffness had a higher impact. In yet another study, using BMP-2 modified poly(acrylamide-co-acrylic acid) hydrogels, BMP-2 was found to have little or no effect towards stem cell differentiation if grafted on softer gels[101]. Hence the mechanical properties of the substrate dictated the potential for stem cell differentiation than the biochemical reagents, explaining the diversity of stem cell behaviors. Efforts to leverage approaches to explore a wider range of combinatorial environments and biochemical pathways, in a systematic manner is necessary, such that the results obtained by different kinds of material systems can be compared. In yet another study, CG scaffolds were prepared by cross-linking treatment *via* dehydrothermal and 1-ethyl-3-3-dimethyl aminopropylcarbodiimide methods, with varying stiffness of 0.5 to 1.5 kPa[102]. MSCs differentiation studies on these scaffolds, showed higher level of RUNX2 expression on the stiffer scaffolds (1.5 kPa) indicating MSCs differentiation to osteogenic lineage. Chondroitin sulphate and hyaluronic acid was found to have a positive influence towards the MSCs differentiation towards osteogenic and chondrogenic lineages, respectively.

Actin filaments of cells are structures for force transmission in response to the substrate with which it comes in contact[103,104]. Increased cell spreading in response to geometric features of the substrate, could result in enhanced actomyosin contractility associated with osteogenic differentiation[103]. The expression of osteogenic markers was demonstrated as stiffness dependent, with maximum osteogenesis observed for fibronectin coated micropatterned polyacrylamide gels with stiffness of 30 kPa.

Studies on the rate of proliferation of mouse ESCs suggest higher proliferation on stiffer substrates of polyacrylamide hydrogels compared to the proliferation on soft substrates, favoring mESC colonization with high levels of Oct3/4 and the Nanog biomarker production[105]. In maintaining the stemness of mESCs on soft hydrogels with varying topography, no significant advantages were observed, suggesting the insensitiveness of topography effect towards mESC stemness. However, surface topography might have a role on substrates with hexagonal or pillar shapes topology or even on stiffer surfaces. It is also believed that either stiffness or topography might play an independent decision factor towards colony formation on 3D substrates. Geometric control of osteogenesis was also studied on polyacrylamide gels prepared by microcontact printing of adhesion proteins where cell spreading and cell geometry are suggested to have some influence towards MSCs differentiation[106]. Stiffer matrices can assist to increase cell spreading, thus promote osteogenesis through enhanced actomyosin contractility and this was observed up to a stiffness of 30 kPa. However, the density of seeded cells were described to over-ride the effect of gel stiffness to cause osteogenic differentiation, according to Xue *et al*[107]. However, this effect was not pronounced on soft gel, where chondrogenic differentiation was induced. Overall, their study highlights the importance of cell-matrix and cell-cell interactions in causing MSCs differentiation. Substrate stiffness was also found to play a predominant role in regulating the growth of rat BM-MSCs, while substrate topography manipulate the cell morphology and spreading, with spherically shaped cells were observed on pillar substrates but not on grooved substrate[108].

In general, studies using mechano-variant polyacrylamide substrates showed that the osteogenic differentiation reduced on substrates with low stiffness and ligand density. However, compared to fibronectin, BMSCs were demonstrated to have a higher binding affinity towards an equivalent bulk of collagen (Figure 5). Such comparisons were further delineated by Sharma *et al*[109], and they found the occurrence of osteogenic differentiation on fibronectin substrates, while tenoblast gene expression was only found on collagen substrates. Two entirely different microsystems namely, sponges and hydrogels were compared by Zhang *et al*[110] where they studied the behavior of MSCs and they found fast cell proliferation in sponges than in hydrogels. Sponges might have encouraged cell-cell and cell-matrix interaction and upon inducing them with media containing TGF-β3, the chondrogenic differentiation of MSCs were better compared to those in hydrogels.

### *Nerve*

From Figure 4 and the abovementioned researches, it can be indicated that for neural differentiation, scaffolds with relatively small stiffness is required. The fate of MSCs towards neural or glial lineages could be manipulated by adjusting the substrate stiffness. Three dimensional collagen-HA scaffolds (fabricated by lyophilization) with higher elasticity (E approximately 1 kPa) induced MSCs into neuronal differentiation, while those with less elasticity (E approximately 10 kPa) induced MSCs into glial differentiation. The elasticity was adjusted by using different concentration

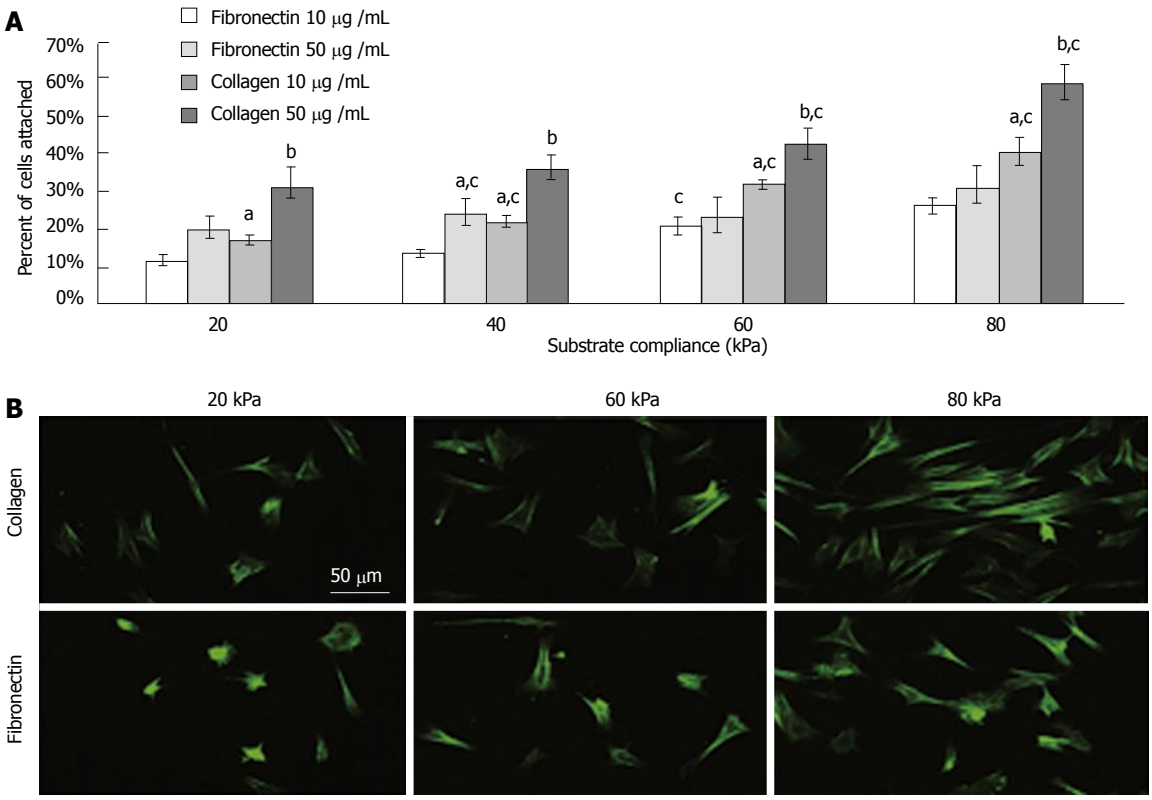


**Figure 5 (A) As the rigidity increased, so did cell attachment. Collagen elicited a greater degree of cell attachment, and increased bulk density of the ligand further enhanced this (B) Morphology on functionalized substrates at 8000 cells/cm$^2$ for 24 h.** [a]statistical difference compared to fibronectin at 10 mg/mL, [b]difference compared to collagen at 10 mg/mL, [c]difference compared to preceding substrate compliance. Cells were stained with FITC-phalloidin. (Scale bar represents 50 μm). Reproduced with permission from Sharma *et al*[109].

of the crosslinking agent, namely 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC)[111]. Tuning the modulus of hydrogels affects neurite outgrowth and ESCs differentiation; with higher modulus gels offered better substrate for neural differentiation and neurite outgrowth, while less modulus enhanced astrocyte formation and cell migration. Thus, ESCs differentiation into neural and glial lineages is influenced by the type, composition, architecture and stiffness of the matrix and the type and concentration of signaling molecules comprehensively[112]. Neuronal differentiation was favored on softer surfaces such as the photopolymerizablemethacrylamide chitosan hydrogel with Young's modulus less than 1000 Pa as confirmed by both immunohistochemistry and qRT-PCR. Oligodendrocyte differentiation was favored on stiffer scaffolds with Young's modulus more than 7000 Pa. However, myelin oligodendrocyte glycoprotein gene expression suggested that oligodendrocyte maturation and myelination was best on scaffolds with modulus less than 1000 Pa where more mature neurons were present. Astrocyte differentiation was only observed on scaffolds with modulus less than 1000 Pa and 3500 Pa and represented less than 2% of the total cell population[113]. Elastic moduli, even in the range of neural tissue, would have a great impact on adult neural stem cell differentiation. The adult NSCs expressed a high level of the neuronal marker, β-tubulin III, on variable moduli interpenetrating polymer networks of 500 Pa, which is similar to the physiological stiffness of brain tissue. Furthermore, under mixed differentiation conditions with serum, softer gels (approximately 100-500 Pa) greatly favored neurons, whereas harder gels (approximately 1000-10000 Pa) promoted glial cultures[114].

To achieve the scaffold with favorable stiffness for nerve tissue regeneration, the fabrication method and the materials should be carefully considered. Hydrogels possess low modulus, and they are suitable as substrates for neuronal differentiation of stem cells. Alternatively, polymers with low modulus might be appropriate for this purpose. Within the category of the neuronal tissue structures, glial differentiation of stem cells might be gained on stiffer scaffolds, and neural differentiation can be achieved on softer scaffolds. By fine tuning the stiffness of the matrix, there is a great possibility to control the differentiation of stem cells into appropriate nerve cells.

### *Cardio-vascular*

Lineage commitment of MSCs towards specific vascular cells (MSCs to ECs or MSCs to SMCs) on electrospun scaffolds could be manipulated by controlling the substrate modulus, from 2 to 15 kPa. Around 95% of MSCs seeded on soft scaffolds (3 kPa) showed Flk-1 endothelial markers within 24 h, while only 20% of

MSCs seeded on the rigid scaffolds (> 8 kPa) showed Flk-1 marker. In contrast, about 80% of MSCs seeded on rigid scaffolds (> 8 kPa) showed smooth muscle α-actin marker within 24 h, while fewer than 10% of MSCs seeded on soft scaffold (< 5 kPa) showed α-actin markers[115]. Besides, the synergistic effect of scaffold elasticity and growth factors has been studied for the differentiation of MSCs. In one such study, MSCs were seeded on soft nanofibrousmatrices with or without VEGF, and in Petri dishes with or without VEGF, where these researchers found that MSCs in soft matrices with VEGF showed significant increase in the expression of endothelia markers (vWF, eNOS, Flt-1, and Flk-1), with faster up-regulation of the endothelial markers. The results indicate that it is critical to control both mechanical factors and biochemical factors to regulate vascular endothelial regeneration of MSCs[116]. MSCs gained very low rate of cardiogenic differentiation after transplantation to infarcted heart, partly because stiffer scar tissue lack the capacity to support cardiogenic differentiation. Thermosensitive and injectable hydrogels with different moduli (16, 45 and 65 kPa) were achieved by controlling the concentration of the hydrogel solution. After 14 d, more than 76% MSCs differentiated into cardiac cells in gels with 45 and 65 kPa, while MSCs in the 65 kPa gel had the highest differentiation efficiency[117]. But the effect of stiffness towards the differentiation of stem cells might be time-dependent. Human embryonic stem cells are sensitive to substrate stiffness during early mesodermal specification, but not late fate choices during cardiac differentiation, when they are seeded on polyacrylamide hydrogels. An intermediate stiffness was most beneficial for cardiomyogenic differentiation than soft or stiff substrates[118].

## CONCLUSION

Selection of appropriate biomaterials for scaffold fabrication and their modification by incorporation of biological constitutes, or by patterning, thus altering the topography and mechanical properties of the scaffolds, might have a significant effect on stem cells behavior. Major literature review shows that MSCs are in favor of soft substrate to differentiate to neuronal cells, with moderately stiffer materials promote myogenic differentiation and more stiffer substrates support osteogenic differentiation, suggesting the effect of substrate mechanical properties in influencing the differentiation of stem cells toward specific lineages. The stiffness of mature tendon range between 500-1000 MPa and bone falls in the range of 20 GPa and this might be a reason for such specific behaviors of stem cells on substrates with proportionate mechanical properties. Prioritizing the stiffness of the substrate alone might not serve as a smart approach, but the presence of specific ligands along with the matrix mechanical compliance, might assist for more specific stem cell differentiations. There exists great difficulty in comparing the results obtained by different research groups, mainly because the parameters such as the ligand type, their concentrations, surface topology or matrix type and their mechanics, and even the cell numbers differ between studies. Hence more uniform and systematically organized studies, are required for a thorough understanding of the stem cell differentiation effect. The extent of differentiation or the % of differentiated cells is another parameter that deserves great attention, such that the cell-substrate can be used for its maximum potential.

The cells on a 2-D platform might be less sensitive to mechanical properties than when they are in 3-D environment. The 3-D structures might provide an additional dimension to mechanical properties, such that the cell related factors such as the integrin ligation, cell contraction, and associated intracellular signaling might be influenced.

## REFERENCES


1 **Preston SL**, Alison MR, Forbes SJ, Direkze NC, Poulsom R, Wright NA. The new stem cell biology: something for everyone. *Mol Pathol* 2003; **56**: 86-96 [PMID: 12665626]

2 **Prabhakaran MP**, Venugopal J, Ghasemi-Mobarakeh L, Kai D, Jin G, Ramakrishna S. Stem cells and nanostructures for advanced tissue regeneration. Biomedical applications of polymeric nanofibers. Berlin: Springer, 2012: 21-62 [DOI: 10.1007/12_2011_113]

3 **Li Y**, Rodrigues J, Tomás H. Injectable and biodegradable hydrogels: gelation, biodegradation and biomedical applications. *Chem Soc Rev* 2012; **41**: 2193-2221 [PMID: 22116474 DOI: 10.1039/C1CS15203C]

4 **Grayson WL**, Martens TP, Eng GM, Radisic M, Vunjak-Novakovic G. Biomimetic approach to tissue engineering Proceedings of the Seminars in cell and developmental biology. Amsterdam: Elsevier, 2009: 665-673 [DOI: 10.1016/j.semcdb.2008.12.008]

5 **Wang A**, Tang Z, Park IH, Zhu Y, Patel S, Daley GQ, Li S. Induced pluripotent stem cells for neural tissue engineering. *Biomaterials* 2011; **32**: 5023-5032 [PMID: 21514663 DOI: 10.1016/j.biomaterials.2011.03.070]

6 **Dehghani L**, Farokhpour M, Karbalaie K, Nematollahi M, Tanhaie S, Hayati-Rodbari N, Kiani-Esfahani A, Hescheler J, Nasr-Esfahani MH, Baharvand H. The influence of dexamethasone administration on the protection against doxorubicin-induced cardiotoxicity in purified embryonic stem cell-derived cardiomyocytes. *Tissue Cell* 2013; **45**: 101-106 [PMID: 23141520 DOI: 10.1016/j.tice.2012.09.009]

7 **Dehghani L**, Hashemi-Beni B, Poorazizi E, Khorvash F, Shaygannejad V, Sedghi M, Vesal S, Meamar R. Evaluation of neural gene expression in serum treated embryonic stem cells in Alzheimer's patients. *J Res Med Sci* 2013; **18**: S20-S23 [PMID: 23961278]

8 **Tabar V**, Studer L. Pluripotent stem cells in regenerative medicine: challenges and recent progress. *Nat Rev Genet* 2014; **15**: 82-92 [PMID: 24434846 DOI: 10.1038/nrg3563]

9 **Eberli D**, Atala A. Tissue engineering using adult stem cells. *Methods in enzymology* 2006; **420**: 287-302 [DOI: 10.1016/S0076-6879(06)20013-2]

10 **Polak JM**, Bishop AE. Stem cells and tissue engineering: past, present, and future. *Ann N Y Acad Sci* 2006; **1068**: 352-366 [PMID: 16831937 DOI: 10.1196/annals.1346.001]

11 **Rammal H**, Harmouch C, Lataillade JJ, Laurent-Maquin D, Labrude P, Menu P, Kerdjoudj H. Stem cells: a promising source for vascular regenerative medicine. *Stem Cells Dev* 2014; **23**: 2931-2949 [PMID: 25167472 DOI: 10.1089/scd.2014.0132]

12 **Riha GM**, Lin PH, Lumsden AB, Yao Q, Chen C. Review: application of stem cells for vascular tissue engineering. *Tissue*

*Eng* 2005; **11**: 1535-1552 [PMID: 16259608 DOI: 10.1089/ten.2005.11.1535]

13 **Yamzon JL**, Kokorowski P, Koh CJ. Stem cells and tissue engineering applications of the genitourinary tract. *Pediatr Res* 2008; **63**: 472-477 [PMID: 18427290 DOI: 10.1203/PDR.0b013e31816a704a]

14 **Yamout B**, Hourani R, Salti H, Barada W, El-Hajj T, Al-Kutoubi A, Herlopian A, Baz EK, Mahfouz R, Khalil-Hamdan R, Kreidieh NM, El-Sabban M, Bazarbachi A. Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. *J Neuroimmunol* 2010; **227**: 185-189 [PMID: 20728948 DOI: 10.1016/j.jneuroim.2010.07.013]

15 **Cohen JA**. Mesenchymal stem cell transplantation in multiple sclerosis. *J Neurol Sci* 2013; **333**: 43-49 [PMID: 23294498 DOI: 10.1016/j.jns.2012.12.009]

16 **Curtis MW**, Russell B. Cardiac tissue engineering. *The J of cardiovascular nursing* 2009; **24**: 87 [DOI: 10.1097/01.JCN.0000343562.06614.49]

17 **Butler DL**, Juncosa-Melvin N, Boivin GP, Galloway MT, Shearn JT, Gooch C, Awad H. Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. *J Orthop Res* 2008; **26**: 1-9 [PMID: 17676628 DOI: 10.1002/jor.20456]

18 **Caplan AI**. The mesengenic process. *Clin Plast Surg* 1994; **21**: 429-435 [PMID: 7924141]

19 **Raghunath J**, Salacinski HJ, Sales KM, Butler PE, Seifalian AM. Advancing cartilage tissue engineering: the application of stem cell technology. *Curr Opin Biotechnol* 2005; **16**: 503-509 [PMID: 16153817 DOI: 10.1016/j.copbio.2005.08.004]

20 **Tuan RS**. Stemming cartilage degeneration: adult mesenchymal stem cells as a cell source for articular cartilage tissue engineering. *Arthritis Rheum* 2006; **54**: 3075-3078 [PMID: 17009225 DOI: 10.1002/art.22148]

21 **Campbell GR**, Campbell JH. Development of tissue engineered vascular grafts. *Curr Pharm Biotechnol* 2007; **8**: 43-50 [PMID: 17311552 DOI: 10.2174/138920107779941426]

22 **Khan WS**, Longo UG, Adesida A, Denaro V. Stem cell and tissue engineering applications in orthopaedics and musculoskeletal medicine. *Stem Cells Int* 2012; **2012**: 403170 [PMID: 22550506 DOI: 10.1155/2012/403170]

23 **Goshima J**, Goldberg VM, Caplan AI. The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. *Clin Orthop Relat Res* 1991; **(269)**: 274-283 [PMID: 1650657]

24 **Hui JH**, Ouyang HW, Hutmacher DW, Goh JC, Lee EH. Mesenchymal stem cells in musculoskeletal tissue engineering: a review of recent advances in National University of Singapore. *Ann Acad Med Singapore* 2005; **34**: 206-212 [PMID: 15827669]

25 **Stamm C**, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schümichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003; **361**: 45-46 [PMID: 12517467 DOI: 10.1016/S0140-6736(03)12110-1]

26 **Neubauer M**, Hacker M, Bauer-Kreisel P, Weiser B, Fischbach C, Schulz MB, Goepferich A, Blunk T. Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro. *Tissue Eng* 2005; **11**: 1840-1851 [PMID: 16411830 DOI: 10.1089/ten.2005.11.1840]

27 **Reekmans K**, Praet J, Daans J, Reumers V, Pauwels P, Van der Linden A, Berneman ZN, Ponsaerts P. Current challenges for the advancement of neural stem cell biology and transplantation research. *Stem Cell Rev* 2012; **8**: 262-278 [PMID: 21537994 DOI: 10.1007/s12015-011-9266-2]

28 **Harris VK**, Yan QJ, Vyshkina T, Sahabi S, Liu X, Sadiq SA. Clinical and pathological effects of intrathecal injection of mesenchymal stem cell-derived neural progenitors in an experimental model of multiple sclerosis. *J Neurol Sci* 2012; **313**: 167-177 [PMID: 21962795 DOI: 10.1016/j.jns.2011.08.036]

29 **Kassem M**, Kristiansen M, Abdallah BM. Mesenchymal stem cells: cell biology and potential use in therapy. *Basic Clin Pharmacol Toxicol* 2004; **95**: 209-214 [PMID: 15546474 DOI: 10.1111/j.1742-7843.2004.pto950502.x]

30 **Sterodimas A**, de Faria J, Nicaretta B, Pitanguy I. Tissue engineering with adipose-derived stem cells (ADSCs): current and future applications. *J Plast Reconstr Aesthet Surg* 2010; **63**: 1886-1892 [PMID: 19969517 DOI: 10.1016/j.bjps.2009.10.028]

31 **Rodriguez AM**, Elabd C, Amri EZ, Ailhaud G, Dani C. The human adipose tissue is a source of multipotent stem cells. *Biochimie* 2005; **87**: 125-128 [PMID: 15733747 DOI: 10.1016/j.biochi.2004.11.007]

32 **Chai C**, Leong KW. Biomaterials approach to expand and direct differentiation of stem cells. *Mol Ther* 2007; **15**: 467-480 [PMID: 17264853 DOI: 10.1038/sj.mt.6300084]

33 **Santiago LY**, Nowak RW, Peter Rubin J, Marra KG. Peptide-surface modification of poly(caprolactone) with laminin-derived sequences for adipose-derived stem cell applications. *Biomaterials* 2006; **27**: 2962-2969 [PMID: 16445976 DOI: 10.1016/j.biomaterials.2006.01.011]

34 **Awad HA**, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* 2004; **25**: 3211-3222 [PMID: 14980416 DOI: 10.1016/j.biomaterials.2003.10.045]

35 **Atkins HL**, Freedman MS. Hematopoietic stem cell therapy for multiple sclerosis: top 10 lessons learned. *Neurotherapeutics* 2013; **10**: 68-76 [PMID: 23192675 DOI: 10.1007/s13311-012-0162-5]

36 **Richards LJ**, Kilpatrick TJ, Bartlett PF. De novo generation of neuronal cells from the adult mouse brain. *Proc Natl Acad Sci USA* 1992; **89**: 8591-8595 [PMID: 1528866 DOI: 10.1073/pnas.89.18.8591]

37 **Reynolds BA**, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; **255**: 1707-1710 [PMID: 1553558 DOI: 10.1126/science.1553558]

38 **Robertson MJ**, Gip P, Schaffer DV. Neural stem cell engineering: directed differentiation of adult and embryonic stem cells into neurons. *Front Biosci* 2008; **13**: 21-50 [PMID: 17981526]

39 **Jäger M**, Feser T, Denck H, Krauspe R. Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers in vitro. *Ann Biomed Eng* 2005; **33**: 1319-1332 [PMID: 16240081 DOI: 10.1007/s10439-005-5889-2]

40 **Saha K**, Pollock JF, Schaffer DV, Healy KE. Designing synthetic materials to control stem cell phenotype. *Curr Opin Chem Biol* 2007; **11**: 381-387 [PMID: 17669680 DOI: 10.1016/j.cbpa.2007.05.030]

41 **Hwang NS**, Varghese S, Li H, Elisseeff J. Regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells in PEG-ECM hydrogels. *Cell Tissue Res* 2011; **344**: 499-509 [PMID: 21503601 DOI: 10.1007/s00441-011-1153-2]

42 **Elisseeff J**, Ferran A, Hwang S, Varghese S, Zhang Z. The role of biomaterials in stem cell differentiation: applications in the musculoskeletal system. *Stem Cells Dev* 2006; **15**: 295-303 [PMID: 16846368 DOI: 10.1089/scd.2006.15.295]

43 **Hwang NS**, Varghese S, Elisseeff J. Controlled differentiation of stem cells. *Advanced drug delivery reviews* 2008; **60**: 199-214 [DOI: 10.1016/j.addr.2007.08.036]

44 **Low WC**, Rujitanaroj PO, Lee DK, Messersmith PB, Stanton LW, Goh E, Chew SY. Nanofibrous scaffold-mediated REST knockdown to enhance neuronal differentiation of stem cells. *Biomaterials* 2013; **34**: 3581-3590 [PMID: 23415645 DOI: 10.1016/j.biomaterials.2013.01.093]

45 **Ayala R**, Zhang C, Yang D, Hwang Y, Aung A, Shroff SS, Arce FT, Lal R, Arya G, Varghese S. Engineering the cell-material interface for controlling stem cell adhesion, migration, and differentiation. *Biomaterials* 2011; **32**: 3700-3711 [PMID: 21396708 DOI: 10.1016/j.biomaterials.2011.02.004]

46 **Shah S**, Yin PT, Uehara TM, Chueng ST, Yang L, Lee KB. Guiding stem cell differentiation into oligodendrocytes using graphene-nanofiber hybrid scaffolds. *Adv Mater* 2014; **26**: 3673-3680 [PMID: 24668911 DOI: 10.1002/adma.201400523]

47 **Dawson E**, Mapili G, Erickson K, Taqvi S, Roy K. Biomaterials for

stem cell differentiation. *Advanced drug delivery reviews* 2008; **60**: 215-228 [DOI: 10.1016/j.addr.2007.08.037]

48 **Boehler RM**, Graham JG, Shea LD. Tissue engineering tools for modulation of the immune response. *Biotechniques* 2011; **51**: 239-240, 242, 244 passim [PMID: 21988690 DOI: 10.2144/000113754]

49 **Parenteau-Bareil R**, Gauvin R, Berthod F. Collagen-based biomaterials for tissue engineering applications. *Materials* 2010; **3**: 1863-1887 [DOI: 10.3390/ma3031863]

50 **Gorgieva S**, Kokol V. Collagen-vs. Gelatine-based biomaterials and their biocompatibility: Review and perspectives. *Biomaterials Applications for Nanomedicine* 2011: 17-58 [DOI: 10.5772/24118]

51 **Lynn AK**, Yannas IV, Bonfield W. Antigenicity and immunogenicity of collagen. *J Biomed Mater Res B Appl Biomater* 2004; **71**: 343-354 [PMID: 15386396 DOI: 10.1002/jbm.b.30096]

52 **Meinel L**, Karageorgiou V, Fajardo R, Snyder B, Shinde-Patil V, Zichner L, Kaplan D, Langer R, Vunjak-Novakovic G. Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Ann Biomed Eng* 2004; **32**: 112-122 [PMID: 14964727]

53 **Koch TG**, Moroni L, Leysi-Derilou Y, Berg LC. Joint cartilage tissue engineering and pre-clinical safety and efficacy testing. InTech, 2011 [DOI: 10.5772/24096]

54 **Ravichandran R**, Venugopal JR, Sundarrajan S, Mukherjee S, Ramakrishna S. Precipitation of nanohydroxyapatite on PLLA/PBLG/Collagen nanofibrous structures for the differentiation of adipose derived stem cells to osteogenic lineage. *Biomaterials* 2012; **33**: 846-855 [PMID: 22048006 DOI: 10.1016/j.biomaterials.2011.10.030]

55 **Nelea V**, Luo L, Demers CN, Antoniou J, Petit A, Lerouge S, R Wertheimer M, Mwale F. Selective inhibition of type X collagen expression in human mesenchymal stem cell differentiation on polymer substrates surface-modified by glow discharge plasma. *J Biomed Mater Res A* 2005; **75**: 216-223 [PMID: 16044417 DOI: 10.1002/jbm.a.30402]

56 **Hosseinkhani H**, Hosseinkhani M, Gabrielson NP, Pack DW, Khademhosseini A, Kobayashi H. DNA nanoparticles encapsulated in 3D tissue-engineered scaffolds enhance osteogenic differentiation of mesenchymal stem cells. *J Biomed Mater Res A* 2008; **85**: 47-60 [PMID: 17688252 DOI: 10.1002/jbm.a.31327]

57 **Battista S**, Guarnieri D, Borselli C, Zeppetelli S, Borzacchiello A, Mayol L, Gerbasio D, Keene DR, Ambrosio L, Netti PA. The effect of matrix composition of 3D constructs on embryonic stem cell differentiation. *Biomaterials* 2005; **26**: 6194-6207 [PMID: 15921736 DOI: 10.1016/j.biomaterials.2005.04.003]

58 **Costa-Pinto AR**, Vargel I, Tuzlakoglu K, Correlo VM, Sol PC, Faria S, Piskin E, Reis RL, Neves NM. Influence of scaffold composition over in vitro osteogenic differentiation of hBMSCs and in vivo inflammatory response. *J Biomater Appl* 2014; **28**: 1430-1442 [PMID: 24255003 DOI: 10.1177/0885328213512385]

59 **Liu X**, Feng Q, Bachhuka A, Vasilev K. Surface modification by allylamine plasma polymerization promotes osteogenic differentiation of human adipose-derived stem cells. *ACS Appl Mater Interfaces* 2014; **6**: 9733-9741 [PMID: 24893152 DOI: 10.1021/am502170s]

60 **Wang M**, Cheng X, Zhu W, Holmes B, Keidar M, Zhang LG. Design of biomimetic and bioactive cold plasma-modified nanostructured scaffolds for enhanced osteogenic differentiation of bone marrow-derived mesenchymal stem cells. *Tissue Eng Part A* 2014; **20**: 1060-1071 [PMID: 24219622 DOI: 10.1089/ten.TEA.2013.0235]

61 **You M**, Peng G, Li J, Ma P, Wang Z, Shu W, Peng S, Chen GQ. Chondrogenic differentiation of human bone marrow mesenchymal stem cells on polyhydroxyalkanoate (PHA) scaffolds coated with PHA granule binding protein PhaP fused with RGD peptide. *Biomaterials* 2011; **32**: 2305-2313 [PMID: 21190731 DOI: 10.1016/j.biomaterials.2010.12.009]

62 **Chua KN**, Chai C, Lee PC, Tang YN, Ramakrishna S, Leong KW, Mao HQ. Surface-aminated electrospun nanofibers enhance adhesion and expansion of human umbilical cord blood hematopoietic stem/progenitor cells. *Biomaterials* 2006; **27**: 6043-6051 [PMID: 16854459 DOI: 10.1016/j.biomaterials.2006.06.017]

63 **Rentsch C**, Hess R, Rentsch B, Hofmann A, Manthey S, Scharnweber D, Biewener A, Zwipp H. Ovine bone marrow mesenchymal stem cells: isolation and characterization of the cells and their osteogenic differentiation potential on embroidered and surface-modified polycaprolactone-co-lactide scaffolds. *In Vitro Cell Dev Biol Anim* 2010; **46**: 624-634 [PMID: 20490706 DOI: 10.1007/s11626-010-9316-0]

64 **Rim NG**, Kim SJ, Shin YM, Jun I, Lim DW, Park JH, Shin H. Mussel-inspired surface modification of poly(L-lactide) electrospun fibers for modulation of osteogenic differentiation of human mesenchymal stem cells. *Colloids Surf B Biointerfaces* 2012; **91**: 189-197 [PMID: 22118890 DOI: 10.1016/j.colsurfb.2011.10.057]

65 **Uebersax L**, Merkle HP, Meinel L. Insulin-like growth factor I releasing silk fibroin scaffolds induce chondrogenic differentiation of human mesenchymal stem cells. *J Control Release* 2008; **127**: 12-21 [PMID: 18280603 DOI: 10.1016/j.jconrel.2007.11.006]

66 **Benoit DS**, Durney AR, Anseth KS. The effect of heparin-functionalized PEG hydrogels on three-dimensional human mesenchymal stem cell osteogenic differentiation. *Biomaterials* 2007; **28**: 66-77 [PMID: 16963119 DOI: 10.1016/j.biomaterials.2006.08.033]

67 **Yang XB**, Roach HI, Clarke NM, Howdle SM, Quirk R, Shakesheff KM, Oreffo RO. Human osteoprogenitor growth and differentiation on synthetic biodegradable structures after surface modification. *Bone* 2001; **29**: 523-531 [PMID: 11728922 DOI: 10.1016/S8756-3282(01)00617-2]

68 **Re'em T**, Tsur-Gang O, Cohen S. The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFbeta1-induced chondrogenesis of human mesenchymal stem cells. *Biomaterials* 2010; **31**: 6746-6755 [PMID: 20542332 DOI: 10.1016/j.biomaterials.2010.05.025]

69 **Paletta JR**, Bockelmann S, Walz A, Theisen C, Wendorff JH, Greiner A, Fuchs-Winkelmann S, Schofer MD. RGD-functionalisation of PLLA nanofibers by surface coupling using plasma treatment: influence on stem cell differentiation. *J Mater Sci Mater Med* 2010; **21**: 1363-1369 [PMID: 19943087 DOI: 10.1007/s10856-009-3947-2]

70 **Kuo YC**, Yeh CF. Effect of surface-modified collagen on the adhesion, biocompatibility and differentiation of bone marrow stromal cells in poly(lactide-co-glycolide)/chitosan scaffolds. *Colloids Surf B Biointerfaces* 2011; **82**: 624-631 [PMID: 21074381 DOI: 10.1016/j.colsurfb.2010.10.032]

71 **Yang Z**, Wu Y, Li C, Zhang T, Zou Y, Hui JH, Ge Z, Lee EH. Improved mesenchymal stem cells attachment and in vitro cartilage tissue formation on chitosan-modified poly(L-lactide-co-epsilon-caprolactone) scaffold. *Tissue Eng Part A* 2012; **18**: 242-251 [PMID: 21902611 DOI: 10.1089/ten.TEA.2011.0315]

72 **Budiraharjo R**, Neoh KG, Kang ET. Hydroxyapatite-coated carboxymethyl chitosan scaffolds for promoting osteoblast and stem cell differentiation. *J Colloid Interface Sci* 2012; **366**: 224-232 [PMID: 22024371 DOI: 10.1016/j.jcis.2011.09.072]

73 **Zandén C**, Hellström Erkenstam N, Padel T, Wittgenstein J, Liu J, Kuhn HG. Stem cell responses to plasma surface modified electrospun polyurethane scaffolds. *Nanomedicine* 2014; **10**: 949-958 [PMID: 24524929 DOI: 10.1016/j.nano.2014.01.010]

74 **Lin J**, Lindsey ML, Zhu B, Agrawal CM, Bailey SR. Effects of surface-modified scaffolds on the growth and differentiation of mouse adipose-derived stromal cells. *J Tissue Eng Regen Med* 2007; **1**: 211-217 [PMID: 18038413]

75 **Hu Y**, Cai K, Luo Z, Zhang Y, Li L, Lai M, Hou Y, Huang Y, Li J, Ding X, Zhang B, Sung KL. Regulation of the differentiation of mesenchymal stem cells in vitro and osteogenesis in vivo by microenvironmental modification of titanium alloy surfaces. *Biomaterials* 2012; **33**: 3515-3528 [PMID: 22333987 DOI: 10.1016/j.biomaterials.2012.01.040]

76 **Yin Z**, Chen X, Chen JL, Shen WL, Hieu Nguyen TM, Gao L, Ouyang HW. The regulation of tendon stem cell differentiation by the alignment of nanofibers. *Biomaterials* 2010; **31**: 2163-2175

[PMID: 19995669 DOI: 10.1016/j.biomaterials.2009.11.083]
77 **Yim EK**, Pang SW, Leong KW. Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage. *Exp Cell Res* 2007; **313**: 1820-1829 [PMID: 17428465 DOI: 10.1016/j.yexcr.2007.02.031]
78 **Pan F**, Zhang M, Wu G, Lai Y, Greber B, Schöler HR, Chi L. Topographic effect on human induced pluripotent stem cells differentiation towards neuronal lineage. *Biomaterials* 2013; **34**: 8131-8139 [PMID: 23891397 DOI: 10.1016/j.biomaterials.2013.07.025]
79 **Smith LA**, Liu X, Hu J, Ma PX. The influence of three-dimensional nanofibrous scaffolds on the osteogenic differentiation of embryonic stem cells. *Biomaterials* 2009; **30**: 2516-2522 [PMID: 19176243 DOI: 10.1016/j.biomaterials.2009.01.009]
80 **Ragetly GR**, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis. *Acta Biomater* 2010; **6**: 1430-1436 [PMID: 19861178 DOI: 10.1016/j.actbio.2009.10.040]
81 **Christopherson GT**, Song H, Mao HQ. The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials* 2009; **30**: 556-564 [PMID: 18977025 DOI: 10.1016/j.biomaterials.2008.10.004]
82 **He L**, Liao S, Quan D, Ma K, Chan C, Ramakrishna S, Lu J. Synergistic effects of electrospun PLLA fiber dimension and pattern on neonatal mouse cerebellum C17.2 stem cells. *Acta Biomater* 2010; **6**: 2960-2969 [PMID: 20193781 DOI: 10.1016/j.actbio.2010.02.039]
83 **Subramony SD**, Dargis BR, Castillo M, Azeloglu EU, Tracey MS, Su A, Lu HH. The guidance of stem cell differentiation by substrate alignment and mechanical stimulation. *Biomaterials* 2013; **34**: 1942-1953 [PMID: 23245926 DOI: 10.1016/j.biomaterials.2012.11.012]
84 Bakeine GJ, Ban J, Grenci G, Pozzato A, Zilio SD, Prasciolu M, Businaro L, Tormen M, Ruaro ME. Design, fabrication and evaluation of nanoscale surface topography as a tool in directing differentiation and organisation of embryonic stem-cell-derived neural precursors. *Microelectronic Engineering* 2009; **86**: 1435-1438 [DOI: 10.1016/j.mee.2009.01.032]
85 **Chaubey A**, Ross KJ, Leadbetter RM, Burg KJ. Surface patterning: tool to modulate stem cell differentiation in an adipose system. *J Biomed Mater Res B Appl Biomater* 2008; **84**: 70-78 [PMID: 17455278 DOI: 10.1002/jbm.b.30846]
86 **Tay CY**, Yu H, Pal M, Leong WS, Tan NS, Ng KW, Leong DT, Tan LP. Micropatterned matrix directs differentiation of human mesenchymal stem cells towards myocardial lineage. *Exp Cell Res* 2010; **316**: 1159-1168 [PMID: 20156435 DOI: 10.1016/j.yexcr.2010.02.010]
87 **Shi Z**, Neoh KG, Kang ET, Poh CK, Wang W. Enhanced endothelial differentiation of adipose-derived stem cells by substrate nanotopography. *J Tissue Eng Regen Med* 2014; **8**: 50-58 [PMID: 22628362 DOI: 10.1002/term.1496]
88 **Oh SH**, Kim TH, Im GI, Lee JH. Investigation of pore size effect on chondrogenic differentiation of adipose stem cells using a pore size gradient scaffold. *Biomacromolecules* 2010; **11**: 1948-1955 [PMID: 20690707 DOI: 10.1021/bm100199m]
89 **Teo BK**, Tan GD, Yim EK. The synergistic effect of nanotopography and sustained dual release of hydrophobic and hydrophilic neurotrophic factors on human mesenchymal stem cell neuronal lineage commitment. *Tissue Eng Part A* 2014; **20**: 2151-2161 [PMID: 24932660 DOI: 10.1089/ten.tea.2013.0382]
90 **Gomes ME**, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG. Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. *J Biomed Mater Res A* 2003; **67**: 87-95 [PMID: 14517865 DOI: 10.1002/jbm.a.10075]
91 **Mauney JR**, Sjostorm S, Blumberg J, Horan R, O'Leary JP, Vunjak-Novakovic G, Volloch V, Kaplan DL. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. *Calcif Tissue Int* 2004; **74**: 458-468 [PMID: 14961210 DOI: 10.1007/s00223-003-0104-7]
92 **Vincent L**, Engler AJ. 5.504 - effect of substrate modulus on cell function and differentiation. In: Ducheyne P Comprehensive biomaterials. Oxford: Elsevier, 2011: 51-63
93 **Bhakta G**, Lim ZX, Rai B, Lin T, Hui JH, Prestwich GD, van Wijnen AJ, Nurcombe V, Cool SM. The influence of collagen and hyaluronan matrices on the delivery and bioactivity of bone morphogenetic protein-2 and ectopic bone formation. *Acta Biomater* 2013; **9**: 9098-9106 [PMID: 23871940 DOI: 10.1016/j.actbio.2013.07.008]
94 **Abrahamsson SO**, Lundborg G, Lohmander LS. Recombinant human insulin-like growth factor-I stimulates in vitro matrix synthesis and cell proliferation in rabbit flexor tendon. *J Orthop Res* 1991; **9**: 495-502 [PMID: 2045976 DOI: 10.1002/jor.1100090405]
95 **Gandavarapu NR**, Alge DL, Anseth KS. Osteogenic differentiation of human mesenchymal stem cells on α5 integrin binding peptide hydrogels is dependent on substrate elasticity. *Biomater Sci* 2014; **2**: 352-361 [PMID: 24660057 DOI: 10.1039/C3BM60149H]
96 **Zhao W**, Li X, Liu X, Zhang N, Wen X. Effects of substrate stiffness on adipogenic and osteogenic differentiation of human mesenchymal stem cells. *Mater Sci Eng C Mater Biol Appl* 2014; **40**: 316-323 [PMID: 24857499 DOI: 10.1016/j.msec.2014.03.048]
97 **Sun H**, Zhu F, Hu Q, Krebsbach PH. Controlling stem cell-mediated bone regeneration through tailored mechanical properties of collagen scaffolds. *Biomaterials* 2014; **35**: 1176-1184 [PMID: 24211076 DOI: 10.1016/j.biomaterials.2013.10.054]
98 **Wang LS**, Du C, Chung JE, Kurisawa M. Enzymatically cross-linked gelatin-phenol hydrogels with a broader stiffness range for osteogenic differentiation of human mesenchymal stem cells. *Acta Biomater* 2012; **8**: 1826-1837 [PMID: 22343003 DOI: 10.1016/j.actbio.2012.02.002]
99 **Tan S**, Fang JY, Yang Z, Nimni ME, Han B. The synergetic effect of hydrogel stiffness and growth factor on osteogenic differentiation. *Biomaterials* 2014; **35**: 5294-5306 [PMID: 24703716 DOI: 10.1016/j.biomaterials.2014.02.040]
100 **Banks JM**, Mozdzen LC, Harley BA, Bailey RC. The combined effects of matrix stiffness and growth factor immobilization on the bioactivity and differentiation capabilities of adipose-derived stem cells. *Biomaterials* 2014; **35**: 8951-8959 [PMID: 25085859 DOI: 10.1016/j.biomaterials.2014.07.012]
101 **Zouani OF**, Kalisky J, Ibarboure E, Durrieu MC. Effect of BMP-2 from matrices of different stiffnesses for the modulation of stem cell fate. *Biomaterials* 2013; **34**: 2157-2166 [PMID: 23290467 DOI: 10.1016/j.biomaterials.2012.12.007]
102 **Murphy CM**, Matsiko A, Haugh MG, Gleeson JP, O'Brien FJ. Mesenchymal stem cell fate is regulated by the composition and mechanical properties of collagen-glycosaminoglycan scaffolds. *J Mech Behav Biomed Mater* 2012; **11**: 53-62 [PMID: 22658154 DOI: 10.1016/j.jmbbm.2011.11.009]
103 **Kilian KA**, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proc Natl Acad Sci USA* 2010; **107**: 4872-4877 [PMID: 20194780 DOI: 10.1073/pnas.0903269107]
104 **Yim EK**, Darling EM, Kulangara K, Guilak F, Leong KW. Nanotopography-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of human mesenchymal stem cells. *Biomaterials* 2010; **31**: 1299-1306 [PMID: 19879643 DOI: 10.1016/j.biomaterials.2009.10.037]
105 **Lü D**, Luo C, Zhang C, Li Z, Long M. Differential regulation of morphology and stemness of mouse embryonic stem cells by substrate stiffness and topography. *Biomaterials* 2014; **35**: 3945-3955 [PMID: 24529627 DOI: 10.1016/j.biomaterials.2014.01.066]
106 **Lee J**, Abdeen AA, Huang TH, Kilian KA. Controlling cell geometry on substrates of variable stiffness can tune the degree of osteogenesis in human mesenchymal stem cells. *J Mech Behav Biomed Mater* 2014; **38**: 209-218 [PMID: 24556045 DOI: 10.1016/j.jmbbm.2014.01.009]
107 **Xue R**, Li JY, Yeh Y, Yang L, Chien S. Effects of matrix elasticity and cell density on human mesenchymal stem cells differentiation. *J Orthop Res* 2013; **31**: 1360-1365 [PMID: 23606500 DOI: 10.1002/

jor.22374]
108 **Li Z**, Gong Y, Sun S, Du Y, Lü D, Liu X, Long M. Differential regulation of stiffness, topography, and dimension of substrates in rat mesenchymal stem cells. *Biomaterials* 2013; **34**: 7616-7625 [PMID: 23863454 DOI: 10.1016/j.biomaterials.2013.06.059]
109 **Sharma RI**, Snedeker JG. Biochemical and biomechanical gradients for directed bone marrow stromal cell differentiation toward tendon and bone. *Biomaterials* 2010; **31**: 7695-7704 [PMID: 20656345 DOI: 10.1016/j.biomaterials.2010.06.046]
110 **Zhang J**, Wu Y, Thote T, Lee EH, Ge Z, Yang Z. The influence of scaffold microstructure on chondrogenic differentiation of mesenchymal stem cells. *Biomed Mater* 2014; **9**: 035011 [PMID: 24818859 DOI: 10.1088/1748-6041/9/3/035011]
111 **Her GJ**, Wu HC, Chen MH, Chen MY, Chang SC, Wang TW. Control of three-dimensional substrate stiffness to manipulate mesenchymal stem cell fate toward neuronal or glial lineages. *Acta Biomater* 2013; **9**: 5170-5180 [PMID: 23079022 DOI: 10.1016/j.actbio.2012.10.012]
112 **Kothapalli CR**, Kamm RD. 3D matrix microenvironment for targeted differentiation of embryonic stem cells into neural and glial lineages. *Biomaterials* 2013; **34**: 5995-6007 [PMID: 23694902 DOI: 10.1016/j.biomaterials.2013.04.042]
113 **Leipzig ND**, Shoichet MS. The effect of substrate stiffness on adult neural stem cell behavior. *Biomaterials* 2009; **30**: 6867-6878 [PMID: 19775749 DOI: 10.1016/j.biomaterials.2009.09.002]
114 **Saha K**, Keung AJ, Irwin EF, Li Y, Little L, Schaffer DV, Healy KE. Substrate modulus directs neural stem cell behavior. *Biophys J* 2008; **95**: 4426-4438 [PMID: 18658232 DOI: 10.1529/biophysj.108.132217]
115 **Wingate K**, Bonani W, Tan Y, Bryant SJ, Tan W. Compressive elasticity of three-dimensional nanofiber matrix directs mesenchymal stem cell differentiation to vascular cells with endothelial or smooth muscle cell markers. *Acta Biomater* 2012; **8**: 1440-1449 [PMID: 22266031 DOI: 10.1016/j.actbio.2011.12.032]
116 **Wingate K**, Floren M, Tan Y, Tseng PO, Tan W. Synergism of matrix stiffness and vascular endothelial growth factor on mesenchymal stem cells for vascular endothelial regeneration. *Tissue Eng Part A* 2014; **20**: 2503-2512 [PMID: 24702044 DOI: 10.1089/ten.TEA.2013.0249]
117 **Li Z**, Guo X, Palmer AF, Das H, Guan J. High-efficiency matrix modulus-induced cardiac differentiation of human mesenchymal stem cells inside a thermosensitive hydrogel. *Acta Biomater* 2012; **8**: 3586-3595 [PMID: 22729021 DOI: 10.1016/j.actbio.2012.06.024]
118 **Hazeltine LB**, Badur MG, Lian X, Das A, Han W, Palecek SP. Temporal impact of substrate mechanics on differentiation of human embryonic stem cells to cardiomyocytes. *Acta Biomater* 2014; **10**: 604-612 [PMID: 24200714 DOI: 10.1016/j.actbio.2013.10.033]

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MINIREVIEWS

# Systems biology approach to developing S$^2$RM-based "systems therapeutics" and naturally induced pluripotent stem cells

Greg Maguire, Peter Friedman

Greg Maguire, Peter Friedman, SRM Living Foundry, BioRegenerative Sciences, Inc., San Diego, CA 92014, United States
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Correspondence to: Greg Maguire, PhD, SRM Living Foundry, BioRegenerative Sciences, Inc., 2658 Del Mar Heights Rd #416, San Diego, CA 92014,
United States. gmaguire@srmfoundry.org
Telephone: +1-858-4137272
Fax: +1-877-8929995
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## Abstract

The degree to, and the mechanisms through, which stem cells are able to build, maintain, and heal the body have only recently begun to be understood. Much of the stem cell's power resides in the release of a multitude of molecules, called stem cell released molecules (SRM). A fundamentally new type of therapeutic, namely "systems therapeutic", can be realized by reverse engineering the mechanisms of the SRM processes. Recent data demonstrates that the composition of the SRM is different for each type of stem cell, as well as for different states of each cell type. Although systems biology has been successfully used to analyze multiple pathways, the approach is often used to develop a small molecule interacting at only one pathway in the system. A new model is emerging in biology where systems biology is used to develop a new technology acting at multiple pathways called "systems therapeutics". A natural set of healing pathways in the human that uses SRM is instructive and of practical use in developing systems therapeutics. Endogenous SRM processes in the human body use a combination of SRM from two or more stem cell types, designated as S$^2$RM, doing so under various state dependent conditions for each cell type. Here we describe our approach in using state-dependent SRM from two or more stem cell types, S$^2$RM technology, to develop a new class of therapeutics called "systems therapeutics." Given the ubiquitous and powerful nature of innate S$^2$RM-based healing in the human body, this "systems therapeutic" approach using S$^2$RM technology will be important for the development of anti-cancer therapeutics, antimicrobials, wound care products and procedures, and a number of other therapeutics for many indications.

**Key words:** Stem cell; Paracrine; Growth factors; Pluripotency; Wound healing; Cancer

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**Core tip:** A fundamentally new type of therapeutic, namely "systems therapeutic," can be realized by reverse engineering the mechanisms of the stem cell released molecules (SRM) processes. Recent data demonstrates that the composition of the SRM is different for each type of stem cell, as well as for different states of each cell type. Although systems biology has been successfully used to analyze multiple pathways, the approach is often used to develop a small molecule interacting at only one pathway in the system. A new model is emerging in biology where systems biology is used to develop a new technology acting at

multiple pathways called "systems therapeutics".

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## INTRODUCTION

The initial few days following fertilization of the human egg, all stem cells in the developing egg are able to create any tissue in the human body, *i.e.,* the stem cells are totipotent. However, about four days following the fertilization of a human egg, the stem cells in the blastocyst begin to differentiate and become pluripotent instead of totipotent, thus being able to differentiate into a more limited set of adult cell types[1]. At this point in time, many different stem cell types are beginning to form that will have unique function related to the development, maintenance, and healing of various tissues throughout the body. The degree to which stem cells differentiate into specific adult phenotypes is only recently beginning to be understood. For example, cell types, such as the progenitor cell preadipocyte and adipose-derived mesenchymal stem cells, each of which was previously classified as one cell type, have now been shown to have phenotypic differences depending on the location of the preadipocyte niches or mesenchymal stem cell niches[2,3]. The signaling factors controlling the development and function of the stem cell types, and indeed the signaling factors that each stem cell type releases, are relatively unknown, but progress is being made. For example, we know that adult stem cells release hundreds of types of proteins within the molecular pool[4], called the stem cell released molecules (SRM), and that each cell type will release a unique pool of molecules[5,6]. These molecules in the SRM will prove to be important for developing many types of therapeutics, including, for example, immunoregulators for organ transplantation[7].

The more differentiated the stem cell, the more specialized the SRM will become. Further, that unique pool of molecules from one stem cell type can change in composition, including the types of molecular species, depending on intrinsic and extrinsic regulatory factors. For example, intrinsic factors related to simple passage number of a stem cell will change the composition of the SRM[8,9] and mesenchymal stem cells (MSCs) in different parts of the body will secrete unique pools of SRM[10]. Telomere dysfunction, whether the cause is intrinsic or extrinsic, will change the nature of the SRM[11]. Likewise, when MSCs derived from fat tissue are conditioned with TNF-α, a significant effect on the SRM is observed with an increased release of factors such as Cathepsin L, interleukin (IL)-6, IL-8, monocyte chemotactic protein-1, matrix metalloproteinase (MMPs), and pentraxin-related protein 3[12]. Further, signaling conditions during the immune modulating responses of human MSCs through Toll-like receptors (TLRs) on the MSCs leads to two basic phenotypic changes of the cells (MSC1 and MSC2) and a consequent dramatic difference in their SRM[13]. Phenotypic changes in the MSC are consequent to: (1) low-level exposure of TLR4 agonists that drives hMSCs toward a pro-inflammatory MSC1 phenotype important for early injury responses; and (2) the TLR3 agonist exposure of hMSCs driving the phenotype to an immunosuppressive MSC2 phenotype that is important to later anti-inflammatory responses that help repair the wound. Culture conditions can also have dramatic effects on SRM. A significant increase in SRM [Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), phosphatidylinositol-glycan biosynthesis class F protein, and TGF-β] was observed after subjecting hMSCs to 72 h hypoxia compared with normoxic conditions[14]. Serum deprivation is another model for ischemia, and was shown to increase the secretion of angiogenic factors released by hMSCs, although the results could have been attributed in full, or part, to differences in cell proliferation rates[15]. Glucose levels have been shown to differentially affect the phenotype of endothelial progenitor cells and mesenchymal progenitor cells[16]. Indeed, subtle variations in cell culture conditions can have significant consequences to the phenotype of stem cells[17].

The state of the extracellular matrix in the stem cell niche is also an important regulator of stem cell phenotype, where, for example, the absence of the SPARC protein in the extracellular matrix (ECM) can drive hematopoietic stem cells into a state of quiescence[18]. Antioxidants and FGF-2 were shown to cause rapid proliferation and a retention of stem cell properties in MSCs, and even enhanced their adipogenic and osteogenic potentials[19]. Interestingly, new studies suggest that adult stem cells, and even somatic cells, may exist in a state of dynamic transition between different levels of potency[20,21] that is dependent on many factors, including paracrine and autocrine factors in the SRM from surrounding stem cells in the same niche, and through the physical state of the surrounding stem cell niche[22]. The state of the oxidative stress in the stem cell may be a contributing factor in phenotype, including the state of pluripotency where the antioxidants curcumin and sesamin were shown to decrease oxidative stress and increase pluripotency[23]. Stem cell derived control factors for determining the fate of stem cells and the potency of cells, including the dedifferentiation of somatic cells, their proliferation, and subsequent differentiation, may include growth differentiation factor 11, a protein secreted by bone marrow mesenchymal stem cells[24] that has been shown to be involved in stem cell fate

and proliferation[25], and has recently been shown to induce a number of regenerative effects, including neurogenesis[26]. Recent studies also demonstrate that NANOG, a pluripotency transcription factor in embryonic stem cells, is also present in at least some adult tissues further raising the possibility that a dynamic state of pluripotency is a naturally occurring process in adults[27]. Again, these shifts in the state of pluripotency will have concomitant shifts in the composition of the SRM released from the stem cell.

Given the differentiation of stem cells into distinct phenotypes, each of which releases a distinct pool of molecules with each distinct pool of molecules inducing a specific set of functions, a knowledge base of the secreted factors (SRM) from each stem cell type and the resulting actions from each pool of molecules will be instructive in the development of therapeutics. The resulting therapeutics that can be developed using the combination of many types of molecules has been termed "systems therapeutics"[28]. The "systems therapeutic" approach, where multiple molecule types target multiple pathways, is in contradistinction to the more traditional approach of small molecule development for perturbation of one pathway.

Moreover, as the stem cell types are cultured ex vivo in the laboratory and driven to state-dependent specific phenotypes through genetic, epigenetic, and other state-dependent variables, the concentration and composition of the SRM as a result can be experimentally manipulated for the purposes of therapeutic development. In addition, distinct pools of SRM from two or more stem cell types cultured in state-dependent conditions can be combined into a collective pool of molecules called $S^2$RM, mimicking the collective actions of multiple stem cell types in their native state in the human body.

## TWO OR MORE TYPES OF STEM CELL INDUCE HEALING

There are two basic forms of cell replacement and regeneration: (1) a maintenance function where renewal of damaged cells during tissue homeostasis (homeostatic growth) are restored; and (2) a response to external injury, such as traumatic wounding, burn, ulceration, or surgery. A given healing response will require many processes acting through a well-orchestrated concert of mechanisms and molecules in the given tissue, and the results of these processes depend on many factors, including the developmental age of the organism. Fundamental factors, such as caspases released from cells undergoing apoptosis, will activate both stem cells and progenitor cells in the wound healing process[29], where apoptosis may be a key factor in cell proliferation during tissue regeneration[30]. Wounds occurring in early to mid-gestational fetal skin have been shown to heal through regeneration without the formation of a scar[31], whereas adult wounds heal by a fibroproliferative response that emphasizes repair over regeneration. The complexity of this process, where fetal wounds differ from adult wounds in inflammatory responses, ECM components, growth factor expression and responses, and profiles of gene expression is exemplified by the observation that adult skin in a fetal environment will still exhibit scar formation[32]. The state dependency of stem cells is so critical as shown in diabetes where the adipose stem cell niche in situ is altered, and the stem cells in the diabetic state are compromised in their ability to establish a vascular network both *in vitro* and *in vivo*[33] where glucose itself has profound direct effects on stem cells[34].

Following injury, wound healing begins rapidly and involves resident and migratory stem cell types, ECM, and soluble factors, including SRM. Stem cells and progenitor cells resident in the skin are certainly involved, but recruitment of stem cells from other sources, including bone marrow, is thought to be important[35]. The mechanisms underlying wound healing include: (1) a rapid release of pro-inflammatory mediators; (2) cell to cell, and cell to extracellular matrix interactions that help mediate cell proliferation, migration, and differentiation; (3) a cascade of events including epithelialization, fibroplasia and angiogenesis[36]; (4) contraction of the wound; and (5) remodeling of the tissue. These events begin at the time of physical injury and proceed continuously throughout the process of tissue repair. Although the processes of repair begin immediately after an injury in all tissues, and all wounds proceed with a similar cascade of healing, some tissues, for example, liver, skeletal tissue, and the eye have different forms of regeneration and repair with variations on the underlying mechanisms[37]. Severe injury has been shown to increase the number of circulating stem cells[38,39] and that these stem cells will participate in the wound healing process[6].

At the onset of trauma bone marrow stem cells will sense histamine released from platelets at H1 receptors and change their phenotype to one of releasing more IL-6 and more IL-8. The increased IL-8 will attract polymorphonuclear neutrophil cells, and the increased IL-6 will facilitate their survival through antiapoptotic functions[40]. When the trauma inducing the injury has stopped, and hemostasis is achieved with an immune response activated, the tissue repair phase will then begin[41]. On the third day after wounding the proliferative phase starts and continues for two or more weeks thereafter. Proliferation begins with fibroblast migration and deposition of newly synthesized ECM, elaborating the initial network of tissue built by fibrin and fibronectin. This phase of wound healing can be clinically observed as an abundant formation of granulation tissue. The complex nature of the proliferative phase is briefly described below[42].

First, fibroblasts and myofibroblasts in the tissue surrounding the wound are stimulated to proliferate for 3 d[43]. The fibroblasts and myofibroblasts then migrate from the surrounding tissue into the wound, attracted by soluble factors TGF-β and platelet-derived growth factor (PDGF) that are released by platelets and inflammatory cells[44]. Appearing in the wound on the third day after injury, the accumulation of fibroblasts in the wound requires their phenotypic modulation. Within the wound, the fibroblasts greatly proliferate, producing and locally releasing the matrix proteins hyaluronan, fibronectin, proteoglycans, and type 1 and type 3 procollagen[45]. Abundant new ECM has accumulated at the end of the first week, further supporting cell migration that is essential for the repair process. Fibroblasts then change to a myofibroblast phenotype. The myofibroblast phenotype contains thick actin bundles that extend below the plasma membrane with pseudopodia attaching to fibronectin and collagen in the ECM. Wound contraction, critical to the reparative process by closing the wound margins, then takes place as the actin bundles begin to retract. Any overabundance of unneeded fibroblasts are then eliminated by apoptosis[44].

The three phases of wound healing involve MSCs to varying degrees, whereby, for example, they recruit macrophages to the wound site[46], induce the fibroblast response to injury[47], and remodel the wound site[48], including a preferential release of collagen type Ⅲ at the site[35]. While the MSCs contribute directly to wound repair by releasing molecules such as collagen to the wound[35], the MSCs also act indirectly by releasing an instruction set to other cells thus initiating, for example, progenitor cell migration to the wound[49]. The MSCs are key to the wound's ability to proceed beyond the inflammatory phase and not maintain a chronic wound state. A significant early component of the mechanism of action of MSCs is their attenuation of the inflammatory response. The addition of MSCs to an active immune response decreases secretion of the proinflammatory cytokines TNF-α and interferon-γ (IFN-γ) while simultaneously increasing the production of anti-inflammatory cytokines IL-10 and IL-4. These anti-inflammatory properties of MSCs impart a particular benefit to chronic wound treatment through SRM, given the SRM can restart healing in chronic wounds by advancing the wound past a chronic inflammatory state into the next stage of healing.

Many studies have shown that MSCs possess antimicrobial activity, critical for clearance of infection in the wound. The antimicrobial activity of MSCs is mediated by two mechanisms: (1) direct secretion of antimicrobial factors such as LL-37[50]; and (2) indirect, by secreting immune-modulating SRM that will upregulate the bacterial killing and phagocytosis of immune cells[51]. Further, the phenotype of macrophages can be regulated by MSCs into various M1 and M2 classes directed to either antimicrobial, phagocytic activity, or one of controlling inflammation[52].

Endogenous MSCs migrate to sites of injury in response to chemotactic signals where they can then modulate inflammation, repair damaged tissue, and facilitate tissue regeneration. Furthermore, bone marrow stem cells home to the injury where cells in the wounded area secrete a protease that interacts with collagen matrix to produce a homing agent[53]. Differentiation and paracrine signaling are two key mechanisms used by MSCs for tissue maintenance and repair. While differentiation of MSCs contributes by directly regenerating damaged tissue, the paracrine signaling by MSCs regulates the local cellular responses to injury, including the differentiation process itself. However, studies of exogenous MSCs show that the contribution of differentiation of these stem cells is limited due to poor engraftment and survival of MSCs at the site of injury, whereas the activation of endogenous stem cells by SRM may provide better results for the differentiation pathway[54], Paracrine signaling by MSCs appears to be the primary mechanism for the beneficial effects of MSCs in wound healing, including the reduction of inflammation, enhanced angiogenesis, and induction of cell migration and proliferation[55].

An analyses of the conditioned medium indicate that MSCs secrete many known SRM mediators of tissue repair including growth factors, cytokines, and chemokines, including VEGF, PDGF, bFGF, EGF, keratinocyte growth factor (KGF), and TGF-β. Stem cells are also known to release exosomes[56], and exosomes from mesenchymal stem cells have been shown to contain factors, including miRNA, that switch cancer stem cells into a dormant state[57]. Such a mechanism is important to dampen the cells in a wound from moving into a state of cancer[58]. Many cell types, including epithelial cells, endothelial cells, fibroblasts, and keratinocytes are responsive to MSC paracrine signaling, where a number of different cellular responses including cell survival, proliferation, migration, and gene expression are regulated. The SRM from MSCs acts as a chemoattractant for dermal fibroblasts, macrophages, endothelial cells, and epidermal keratinocytes, *in vitro*. The presence of either MSCs or the SRM from MSCs have been shown to promote wound closure through the activation of dermal fibroblasts. MSCs also secrete mitogens, leading to the proliferation of keratinocytes, dermal fibroblasts, and endothelial cells *in vitro*. Further, dermal fibroblasts secrete increased amounts of collagen type Ⅰ and alter gene expression in response to either MSCs in co-culture or the SRM from MSCs. These data suggest that SRM from MSCs stimulate proliferation and migration of the key cell types in the wound. In addition, the SRM of MSCs imparts anti-scarring properties to wound healing through the secretion of VEGF and hepatocyte growth factor (HGF), and through maintaining a normal balance between

TGF-β1 and TGF-β3. The pathways underlying MSC processes in wound healing are complex, and further details of these processes can be found in recent reviews. Stem cell niches in other regions of the body, including the hematopoietic stem cell niche, appear to be equally complicated as the skin stem cell niche with a rich interaction amongst many cell types, including a number of stem cell types and their respective SRM[59].

## NATURALLY INDUCED PLURIPOTENT STEM CELL WITHIN THE STATE DEPENDENT STEM CELL NICHE

Natually occurring endogenous iPSs, or naturally induced pluripotent stem cells (NiPSs) occur within the state dependent stem cell niche. While induction to a totipotent state has not been realized, dedifferentiation seems to be an important adaptive mechanism in both the animal[60] and plant kingdoms[61] where cells can be induced to become pluripotent. In addition to the therapeutic development of embryonic stem cells and iPSs, the use of adult stem cells and the molecules that they release have been intensively investigated and have current therapeutic applications. Further, SRM from stem cells or other molecules from neighboring cells, such as ciliary neurotrophic factor (CNTF), have been shown to dedifferentiate myoblasts into multipotent progenitor cells. The dedifferentiated myoblasts were then able to differentiate into several new phenotypes[62].

The endogenous mechanisms of adult stem cells, and possibly somatic cells in the stem cell niche, seem to include the ability to reprogram themselves into more primordial states that are pluripotent. That is, the adult stem cell, and even somatic cells, may exist in a state of dynamic transition between different levels of potency that is dependent on many factors, including paracrine and autocrine factors in the SRM from surrounding cells in the stem cell niche, and by the physical, chemical, and electrical state of the stem cell niche[63-65]. Recently, treatment with reversine, a type of purine, transformed 3T3-L1 preadipocytes into MSC-like cells, as evidenced by the expression of MSCs marker genes. The transform allowed differentiation of lineage-committed 3T3-L1 preadipocytes to osteoblasts under the osteogenic condition *in vitro*[66]. Beyond transcription factors contained in the SRM, physical manipulation through the cytoskeleton is known to transmit signals to the chromatin[67] and reprogram cells[68], and may represent an additional biophysical, in addition to biochemical means, for driving cells to varying levels of potency. Reprogramming of differentiated cells to stem-like cells has been described in several tissues and is well studied in the epithelial-mesenchymal transition where a differentiated epithelial cell transforms to a mesenchymal cell with a stem cell-like phenotype. Thus, by understanding adult stem cell function, we may develop the means to use these cells in many ways to maintain and heal the body, including a means of controlling naturally occurring iPSs (NiPSs).

The physical, chemical, and electrical state of the stem cell niche will have profound influences on stem cell function. Alterations of the stem cell niche in diseases such as diabetes will decrease the ability of endogenous stem cells, or autologous administered stem cells, to increase neovascularization and promote wound healing[33].

In Figure 1, we see levels of interactions that may control the natural iPSC state. Considering wound healing as described in the aforementioned section, many factors, such as histamine, an important regulator of cell fate, including neurons[69,70] are released at the site of injury. As an example of the actions of these factors, histamine will activate TRPM4 calcium channels in the mesenchymal stem cells and bias the dynamic transition of the stem cells toward differentiation into the needed mature cells types at the injury site[71], including osteoclastogenesis[72]. Similarly, exposure to sunlight will stimulate vitamin D3 levels and induce differentiation of stem cells, doing so through a downstream pathway that includes histamine[73].

Reprogramming of cells to push the dynamic transition towards more potency has been specifically shown in mammalian cells whereby muscle cells[74] and pancreas cells[75] will dedifferentiate into a more pluripotent state following injury, and where fibroblasts were incubated in the SRM of adipose-derived stem cells. The fibroblasts displayed gene expression that was indicative of pluripotency in which repressive histone modifications were reduced, and increased global demethylation was present. The *Col1a1* and *Col1a2* genes, typically found in differentiated cells only, demonstrated reduced expression, and also demonstrated increased methylation in the 5′-flanking regulatory regions[76]. Of the many factors released by mesenchymal stem cells, microRNA is one of the factors that have been shown to induce pluripotency in mouse and human somatic cells[77]. In general, stress is a key factor that can naturally induce pluripotency. For example, simple isolation of mammalian cells from contact with other cells and their normal niche, originally exhibiting a limited differentiation potential, may become multipotent[78]. Pluripotent cells can reside in the naïve state or the primed state where the naïve state is more potent than the primed state[79]. Dedifferentiation under hypoxic conditions can drive committed cells beyond the primed state fully back to the naive state of potency where the pluripotent cells are then capable of forming teratomas[80].

Cancer cells and pluripotent stem cells follow certain common rules. Both cell types, when placed in a dysregulated extracellular matrix, will exhibit an increased state of potency. Cancer cells, when returned to a regulated ECM, will revert to a normal phenotype[58,81]. Likewise, dedifferentiation of cells into

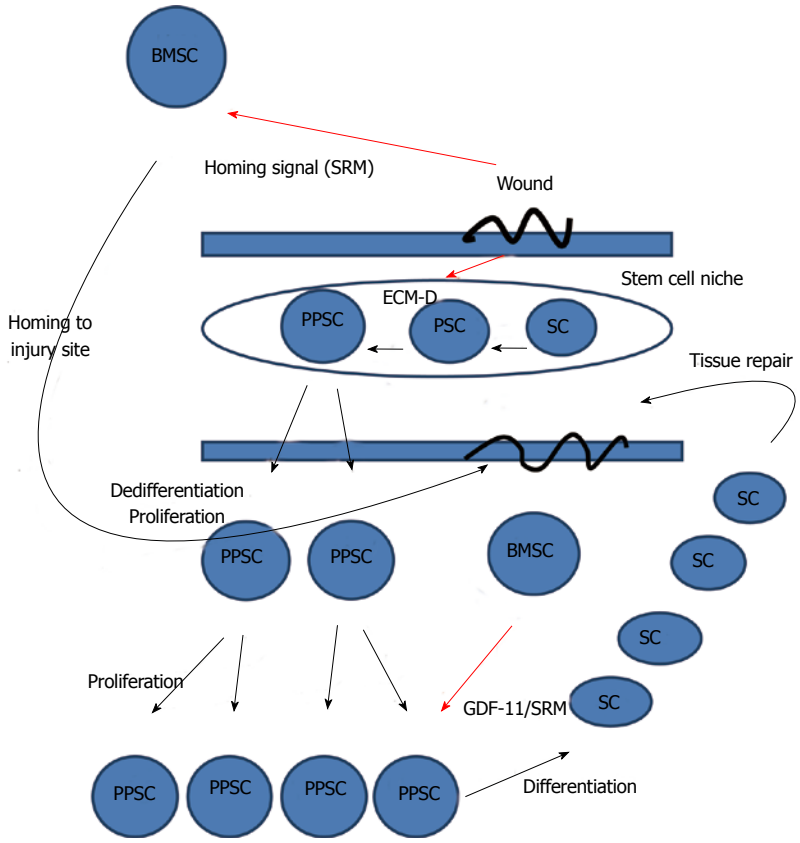


**Figure 1 General model of wound healing.** The wounded state sends a homing signal to bone marrow stem cells and disrupts the ECM. Disruption of the ECM will shift the dynamic transition of potency towards dedifferentiation and the more pluripotent state. The more pluripotent state will cause the cells to proliferate. After proliferation, the migration of bone marrow stem cells to the wound site will release stem cell released molecules, including GDF-11, that stops proliferation and induces differentiation allowing newly differentiated somatic cells to repair the tissue. Thus, in our model, GDF11 is released from BMSCs and is a master regulator of stem cell transcription that inhibits cell proliferation and migration by down-regulating the expression of numerous genes involved in both these processes[93]. ECM-D: Extracellular matrix disruption; SC: Somatic cell; PPSC: Pluripotent stem cell; BMSC: Bone marrow stem cell; PSC: Potent stem cell.

a pluripotent state can occur when the cell is isolated and loses connections with other cells and the ECM[78], and stem cells that have differentiated can revert to a more pluripotent state by changes in the concentration of the ECM associated protein, L-proline[82]. Thus, induction of pluripotent stem cells is a naturally occurring phenomenon that can be controlled *in vivo* for therapeutic effect by manipulating the state of the stem cell niche.

## CONCEPTS OF A SYSTEMS THERAPEUTIC

An abnormality in one pathway, or even at one level of the organism, such as at the level of genes, does not explain a disease. Rather, disease reflects the perturbations of the complex system of biological pathways acted on by a complex set of environmental regulators. Most previous work to understand disease and drug response traits have focused on single dimensions, and even single pathways, of the system. Achieving a more comprehensive and predictive understanding of disease and drug response requires examining living systems in multiple dimensions and at multiple scales. Although biological engineering principles are necessary, with the requirement to remove superfluous complexity for the development of a particular therapeutic, the individual components of complex systems are highly coupled such that the individual components cannot be analyzed in isolation. This predicament in biology, such as the desire to place the sequencing of the genome as the singular predictor of disease, is similar to that dictum in physics where electrodynamics was broken down into the misbegotten particles and fields theorem by Bohr and his Copenhagen interpretation[83]. Biological complexity is an extreme example of complexity, arising from a biological system that includes active, plastic components, nested feedback loops, flexible design principles, component multi-functionality, and multiple layers of system dynamics developed through evolutionary processes that are, at least partially, driven through the downward causation of environmental regulators. The power of the dynamic biological system has been recognized in engineering where, for example, neuromorphic engineering[84] has become an important player in the development of new computer chip technologies such as TrueNorth[85].

Despite the use of systems analysis in the fields

of biology and therapeutic development, therapeutic development has often remained as one using systems biology techniques for finding the one pathway, or the one target, that is best perturbed to develop the therapeutic. "Finding the magic bullet" is a common phrase that describes this common problem. A shift in mindset to one of finding the minimum set of pathways, or the minimum set of targets, using the "minimum molecule set" to perturb these targets in order to best develop a therapeutic is now needed. Thus, biological function results from a system, and a particular disease state is the result of multiple perturbations in that system, not just one perturbation. Therefore, through an understanding of complex pathways in normal and disease states, and using computationally intensive biological design-build-test-analyze cycles, with therapeutic molecule production batches based on this process, we can hope to develop safe and efficacious therapeutics. This will occur through a multi-targeted, "systems therapeutic" approach. The approach then is to use a system of molecules, the minimum molecule set, that is not overly reductionist so as to be ineffective, but instead use the optimal number of molecule species that are sufficient to realize a safe and efficacious therapeutic. Recognizing that diseases are the result of complex interactions among many networks has significant implications for drug discovery, leading to the design of combinations of molecular species that impact entire network states, rather than designing reductionist drugs that target specific genes that are associated, often weakly, with disease.

## DEVELOPMENT OF SYSTEMS ANTIMICROBIALS

The attempt to develop animal-derived antimicrobials is not new. For example, in the 1990s great hope, and many dollars spent, was placed on the development of a small peptide from frog (Xenopus laevis) skin as an antibiotic[86]. The observation that frog skin heals itself, despite the frog living in a very septic environment, led to the formation of Magainin Pharmaceuticals. After years, and millions of dollars, spent on development and Phase II clinical trials, today Magainin's assets are the auction block (Magainin changed names to Genera and then liquidated: http://www.fiercebiotech.com/press-releases/genaera-corporation-announces-approval-plan-liquidation-and-dissolution-board-direc-0). Why? Because the frog's skin does not heal itself through a reductionist approach with only one molecule (a peptide), and Magainin didn't fully learn the frog's lesson. The lesson not learned was that Magainin developed their antibiotic based on one peptide, a reductionist approach, instead of a mix of antimicrobial factors, a systems antimicrobial approach.

Lipids were first demonstrated by Koch[87] to have antibiotic activity, and exists in human skin, for example, as a wide range of molecule types comprising a significant part of the innate immune system[88]. Like Magainin, a similar reductionist approach was used in the development of squalamine, a lipid compound (aminosterol) derived from the dogfish shark (Squalus acanthias). Squalamine was initially discovered on the basis of its anti-bacterial activity, and has broad spectrum antimicrobial activity against fungi, protozoa, and many viruses[89]. Sadly, isolated squalamine was never approved for antimicrobial use and is now sold as a nutritional product by a number of companies in capsule form. Once again, the "Copenhagen reductionist" approach to therapeutic development has failed us. Here again, instead, an approach to developing antimicrobials using a collection of molecules, including peptides and lipids, is in development.

## DEVELOPMENT OF CANCER SYSTEMS THERAPEUTIC

Cancer is strongly associated with a deregulated ECM[58,90]. While cancer and stem cells are regulated by many factors, both cancer cells and pluripotent stem cells follow certain common rules such as regulation by the ECM. Both cell types, when placed in a dysregulated extracellular matrix, will exhibit an increased state of potency. Cancer cells, when returned to a regulated ECM, will revert to a normal phenotype[58,81]. As beautifully explained by Mina Bissell at University of California Berkeley, during development, cells can spatially arrange themselves, differentiate, and change their SRM composition in response to a variety of signals in the microenvironment, including morphogens, biophysical manipulation, juxtacrine signals, and the ECM. All of these components in the microenvironment are sensitive to signals from other tissues and organs of the developing embryo as well as through downward causation from the macroenvironment. However, following organ formation, the microenvironment/ECM integrates and constrains the organ architecture and function, thus ensuring structural and functional homeostasis and therefore, a normal organ phenotype. However, when the organ architecture in adults is insulted by mutations and/or changes in the microenvironment such as ECM dysregulation and/or inflammation, that organ is transformed by the initiation of developmental and embryonic circuits. However, in the adult, the microenvironment is no longer embryonic in nature, and the ECM dysregulation and inflammation leads to a pluripotent state, *i.e.*, the cancerous state. Bissell argues that tumors become new evolutionary organs searching for homeostasis[58]. Recent work fits the paradigm of Bissell, such as that of Liou *et al*[91] who describes the detailed steps that Kras-mutated acinar cells follow as they change into duct-like cells with a more potent state. They observed that Kras proteins in the acinar cells switched on

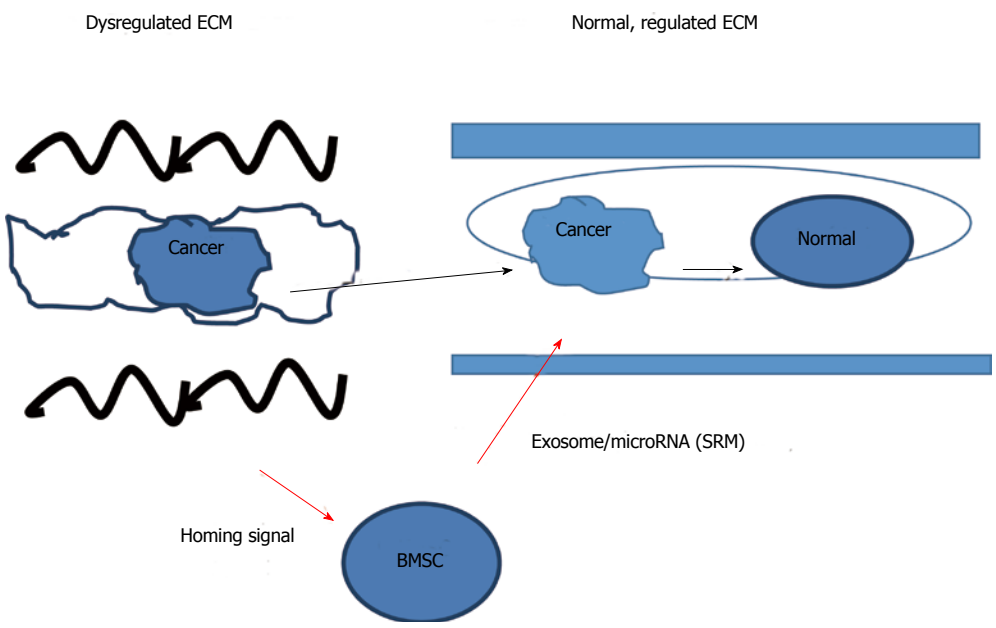


**Figure 2 Regulation of the cancer/pluripotent phenotype by stem cells and extracellular matrix.** The cancer/pluripotent cell phenotype can be regulated by the extracellular matrix (ECM) and stem cells, where cancer cells can be removed from a dysregulated ECM and placed into a normal ECM and the cancer/pluripotent phenotype will revert to a normal, somatic cell phenotype. Likewise, if a dysregulated ECM is reconstructed into a normal state, the cancer/pluripotent phenotype will revert to the normal somatic cell phenotype. Further regulation of the cancer/pluripotent phenotype can be regulated by a number of factors, including microRNA contained within exosomes that were released from mesenchymal stem cells serving to change the state of the cancer cell into one of quiescence. MBSC: BM-stroma cell.

intercellular adhesion molecule-1. This in turn attracts macrophages. The macrophages then release a variety of proteins, including MMPs that degrade ECM. Following dysregulation of ECM, the acinar cells then transform into the stem cell-like phenotype. Thus, the direct link between Kras mutations, the inflammatory environment, and dysregulated ECM that drive the initiation of pancreatic cancer is demonstrated.

Similar to the cancer state, dedifferentiation of cells into a pluripotent state can occur when the cell is isolated and loses connections with other cells and the ECM[78], and stem cells that have differentiated can revert to a more pluripotent state by changes in the concentration of the ECM associated protein, L-proline[82]. Given that that the ECM can act through mechanical and biochemical mechanisms to regulate the cancer phenotype, one important means to revert the cancer phenotype to the normal somatic cell phenotype is to use $S^2$RM technology to reestablish a normal ECM microenvironment for the cancer cell. That is, using one progenitor cell type to release the building blocks of the ECM, such as collagen, and using another stem cell type to release other building blocks and the instruction sets to build the architecture of the ECM, the normal state of the ECM can be rebuilt and lead to the reversion of the cancer cell phenotype to a more normal somatic cell phenotype as depicted in Figure 2. Thus building on the model developed by Mina Bissell, our model suggests that that the microenvironment/ECM, which is largely comprised of, and developed by $S^2$RM, is key to the regulation of the initiation and degree of pluripotency of cells, controls the "stop" signals for driving potency and then initiates differentiation of the pluripotent stem cells. The $S^2$RM thus controls homeostasis whereby the state of dynamic transition between different levels of potency[92] is set to a proper level in which to produce enough new cells to maintain and heal tissue, but not too much so as to allow uncontrolled, cancerous growth of the tissue.

In summary, the $S^2$RM technology provides a natural means for mimicking and stimulating the healing properties of the human body. Instead of using foreign molecules, natural molecules are used that will induce the initiation of natural processes with little or no side-effects. Further, instead of using a small molecule approach where one molecule interacts at one pathway underlying a multi-pathway disease is used, here the $S^2$RM approach uses multiple molecules to perturb multiple pathways underlying the disease, thus yielding a more efficacious result than the one molecule-one pathway reductionist approach.

The $S^2$RM approach will introduce all of the needed molecules to the tissue to induce a full wound healing cascade of events, unlike an approach using the molecules from one stem cell type that will introduce only a portion of the needed molecules and thus provide a fraction of the efficacy that the $S^2$RM provides. And, $S^2$RM uses the particular molecules from the particular stem cells types relevant to the particular tissue to be healed. This is distinct from the "one size fits all" approach where one stem cell type is used to develop therapeutics for the whole body. Therefore, $S^2$RM provides all of the building blocks, such as the different collagen types, to rebuild the tissue, and also provides the instruction set molecules, such as microRNA, that

will deliver the needed architectural commands that will lay the building blocks in their proper places for that particular tissue. During this rebuilding process, the immune response will also be modulated by $S^2$RM, so that inflammation is quelled, allowing the rebuilding to proceed within a normalized framework that is not swollen. The $S^2$RM rebuilding process institutes two fundamental stem cell healing processes: (1) Mimicking the actions of multiple stem cell types and the molecules that they release in the relevant tissue, and (2) reconditioning the endogenous stem cell niche itself and driving the niche to a more primordial, potent state, allowing endogenous stem cell processes to better induce a healing response. Thus, a systems therapeutic approach using multiple molecules from multiple stem cell types called $S^2$RM is used to develop a safer, more natural, and more efficacious therapeutic that both mimics and facilitates the natural adult stem cell healing processes of our body.

## REFERENCES


1 **Cauffman G**, De Rycke M, Sermon K, Liebaers I, Van de Velde H. Markers that define stemness in ESC are unable to identify the totipotent cells in human preimplantation embryos. *Hum Reprod* 2009; **24**: 63-70 [PMID: 18824471 DOI: 10.1093/humrep/den351]

2 **Macotela Y**, Emanuelli B, Mori MA, Gesta S, Schulz TJ, Tseng YH, Kahn CR. Intrinsic differences in adipocyte precursor cells from different white fat depots. *Diabetes* 2012; **61**: 1691-1699 [PMID: 22596050 DOI: 10.2337/db11-1753]

3 **Ong WK**, Tan CS, Chan KL, Goesantoso GG, Chan XH, Chan E, Yin J, Yeo CR, Khoo CM, So JB, Shabbir A, Toh SA, Han W, Sugii S. Identification of specific cell-surface markers of adipose-derived stem cells from subcutaneous and visceral fat depots. *Stem Cell Reports* 2014; **2**: 171-179 [PMID: 24527391 DOI: 10.1016/j.stemcr.2014.01.002]

4 **Sze SK**, de Kleijn DP, Lai RC, Khia Way Tan E, Zhao H, Yeo KS, Low TY, Lian Q, Lee CN, Mitchell W, El Oakley RM, Lim SK. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteomics* 2007; **6**: 1680-1689 [PMID: 17565974 DOI: 10.1074/mcp.M600393-MCP200]

5 **Ribeiro CA**, Fraga JS, Grãos M, Neves NM, Reis RL, Gimble JM, Sousa N, Salgado AJ. The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations. *Stem Cell Res Ther* 2012; **3**: 18 [PMID: 22551705 DOI: 10.1186/scrt109]

6 **Berardis S**, Lombard C, Evraerts J, El Taghdouini A, Rosseels V, Sancho-Bru P, Lozano JJ, van Grunsven L, Sokal E, Najimi M. Gene expression profiling and secretome analysis differentiate adult-derived human liver stem/progenitor cells and human hepatic stellate cells. *PLoS One* 2014; **9**: e86137 [PMID: 24516514 DOI: 10.1371/journal.pone.0086137]

7 **Kato T**, Okumi M, Tanemura M, Yazawa K, Kakuta Y, Yamanaka K, Tsutahara K, Doki Y, Mori M, Takahara S, Nonomura N. Adipose tissue-derived stem cells suppress acute cellular rejection by TSG-6 and CD44 interaction in rat kidney transplantation. *Transplantation* 2014; **98**: 277-284 [PMID: 24983309 DOI: 10.1097/TP.0000000000000230]

8 **Crisostomo PR**, Wang M, Wairiuko GM, Morrell ED, Terrell AM, Seshadri P, Nam UH, Meldrum DR. High passage number of stem cells adversely affects stem cell activation and myocardial protection. *Shock* 2006; **26**: 575-580 [PMID: 17117132 DOI: 10.1097/01.shk.0000235087.45798.93]

9 **Deschene ER**, Myung P, Rompolas P, Zito G, Sun TY, Taketo MM, Saotome I, Greco V. β-Catenin activation regulates tissue growth non-cell autonomously in the hair stem cell niche. *Science* 2014; **343**: 1353-1356 [PMID: 24653033 DOI: 10.1126/science.1248373]

10 **Park CW**, Kim KS, Bae S, Son HK, Myung PK, Hong HJ, Kim H. Cytokine secretion profiling of human mesenchymal stem cells by antibody array. *Int J Stem Cells* 2009; **2**: 59-68 [PMID: 24855521 DOI: 10.15283/ijsc.2009.2.1.59]

11 **Ju Z**, Jiang H, Jaworski M, Rathinam C, Gompf A, Klein C, Trumpp A, Rudolph KL. Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med* 2007; **13**: 742-747 [PMID: 17486088 DOI: 10.1038/nm1578]

12 **Lee MJ**, Kim J, Kim MY, Bae YS, Ryu SH, Lee TG, Kim JH. Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. *J Proteome Res* 2010; **9**: 1754-1762 [PMID: 20184379 DOI: 10.1021/pr900898n]

13 **Waterman RS**, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PLoS One* 2010; **5**: e10088 [PMID: 20436665 DOI: 10.1371/journal.pone.0010088]

14 **Kinnaird T**, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004; **94**: 678-685 [PMID: 14739163 DOI: 10.1161/01.RES.0000118601.37875.AC]

15 **Oskowitz A**, McFerrin H, Gutschow M, Carter ML, Pochampally R. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. *Stem Cell Res* 2011; **6**: 215-225 [PMID: 21421339 DOI: 10.1016/j.scr.2011.01.004]

16 **Keats E**, Khan ZA. Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose. *PLoS One* 2012; **7**: e38752 [PMID: 22701703 DOI: 10.1371/journal.pone.0038752]

17 **Baer PC**. Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype in vivo and in vitro. *World J Stem Cells* 2014; **6**: 256-265 [PMID: 25126376 DOI: 10.4252/wjsc.v6.i3.256]

18 **Ehninger A**, Boch T, Medyouf H, Müdder K, Orend G, Trumpp A. Loss of SPARC protects hematopoietic stem cells from chemotherapy toxicity by accelerating their return to quiescence. *Blood* 2014; **123**: 4054-4063 [PMID: 24833352 DOI: 10.1182/blood-2013-10-533711]

19 **Sun LY**, Pang CY, Li DK, Liao CH, Huang WC, Wu CC, Chou YY, Li WW, Chen SY, Liu HW, Chang YJ, Cheng CF. Antioxidants cause rapid expansion of human adipose-derived mesenchymal stem cells via CDK and CDK inhibitor regulation. *J Biomed Sci* 2013; **20**: 53 [PMID: 23915242 DOI: 10.1186/1423-0127-20-53]

20 **Tata PR**, Mou H, Pardo-Saganta A, Zhao R, Prabhu M, Law BM, Vinarsky V, Cho JL, Breton S, Sahay A, Medoff BD, Rajagopal J. Dedifferentiation of committed epithelial cells into stem cells in vivo. *Nature* 2013; **503**: 218-223 [PMID: 24196716 DOI: 10.1038/nature12777]

21 **Kusaba T**, Lalli M, Kramann R, Kobayashi A, Humphreys BD. Differentiated kidney epithelial cells repair injured proximal tubule. *Proc Natl Acad Sci USA* 2014; **111**: 1527-1532 [PMID: 24127583 DOI: 10.1073/pnas.1310653110]

22 **Su G**, Zhao Y, Wei J, Xiao Z, Chen B, Han J, Chen L, Guan J, Wang R, Dong Q, Dai J. Direct conversion of fibroblasts into neural progenitor-like cells by forced growth into 3D spheres on low attachment surfaces. *Biomaterials* 2013; **34**: 5897-5906 [PMID: 23680365 DOI: 10.1016/j.biomaterials.2013.04.040]

23 **Yanes O**, Clark J, Wong DM, Patti GJ, Sánchez-Ruiz A, Benton HP, Trauger SA, Desponts C, Ding S, Siuzdak G. Metabolic oxidation regulates embryonic stem cell differentiation. *Nat Chem Biol* 2010; **6**: 411-417 [PMID: 20436487 DOI: 10.1038/nchembio.364]

24 **Lai RC**, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010; **4**: 214-222 [PMID:

20138817 DOI: 10.1016/j.scr.2009.12.003]

25 **Gokoffski KK**, Wu HH, Beites CL, Kim J, Kim EJ, Matzuk MM, Johnson JE, Lander AD, Calof AL. Activin and GDF11 collaborate in feedback control of neuroepithelial stem cell proliferation and fate. *Development* 2011; **138**: 4131-4142 [PMID: 21852401 DOI: 10.1242/dev.065870]

26 **Katsimpardi L**, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz GR, Chen JW, Lee RT, Wagers AJ, Rubin LL. Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science* 2014; **344**: 630-634 [PMID: 24797482 DOI: 10.1126/science.1251141]

27 **Piazzolla D**, Palla AR, Pantoja C, Cañamero M, de Castro IP, Ortega S, Gómez-López G, Dominguez O, Megías D, Roncador G, Luque-Garcia JL, Fernandez-Tresguerres B, Fernandez AF, Fraga MF, Rodriguez-Justo M, Manzanares M, Sánchez-Carbayo M, García-Pedrero JM, Rodrigo JP, Malumbres M, Serrano M. Lineage-restricted function of the pluripotency factor NANOG in stratified epithelia. *Nat Commun* 2014; **5**: 4226 [PMID: 24979572 DOI: 10.1038/ncomms5226]

28 **Maguire G**. Systems biology approach to developing "systems therapeutics". *ACS Med Chem Lett* 2014; **5**: 453-455 [PMID: 24900858 DOI: 10.1021/ml5000614]

29 **Li F**, Huang Q, Chen J, Peng Y, Roop DR, Bedford JS, Li CY. Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. *Sci Signal* 2010; **3**: ra13 [PMID: 20179271 DOI: 10.1126/scisignal.2000634]

30 **Fan Y**, Bergmann A. Apoptosis-induced compensatory proliferation. The Cell is dead. Long live the Cell! *Trends Cell Biol* 2008; **18**: 467-473 [PMID: 18774295 DOI: 10.1016/j.tcb.2008.08.001]

31 **Rowlatt U**. Intrauterine wound healing in a 20 week human fetus. *Virchows Arch A Pathol Anat Histol* 1979; **381**: 353-361 [PMID: 155931 DOI: 10.1007/BF00432477]

32 **Longaker MT**, Whitby DJ, Ferguson MW, Lorenz HP, Harrison MR, Adzick NS. Adult skin wounds in the fetal environment heal with scar formation. *Ann Surg* 1994; **219**: 65-72 [PMID: 8297179 DOI: 10.1097/00000658-199401000-00011]

33 **Rennert RC**, Sorkin M, Januszyk M, Duscher D, Kosaraju R, Chung MT, Lennon J, Radiya-Dixit A, Raghvendra S, Maan ZN, Hu MS, Rajadas J, Rodrigues M, Gurtner GC. Diabetes impairs the angiogenic potential of adipose-derived stem cells by selectively depleting cellular subpopulations. *Stem Cell Res Ther* 2014; **5**: 79 [PMID: 24943716 DOI: 10.1186/scrt468]

34 **Cramer C**, Freisinger E, Jones RK, Slakey DP, Dupin CL, Newsome ER, Alt EU, Izadpanah R. Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. *Stem Cells Dev* 2010; **19**: 1875-1884 [PMID: 20380516 DOI: 10.1089/scd.2010.0009]

35 **Fathke C**, Wilson L, Hutter J, Kapoor V, Smith A, Hocking A, Isik F. Contribution of bone marrow-derived cells to skin: collagen deposition and wound repair. *Stem Cells* 2004; **22**: 812-822 [PMID: 15342945 DOI: 10.1634/stemcells.22-5-812]

36 **Tamai K**, Yamazaki T, Chino T, Ishii M, Otsuru S, Kikuchi Y, Iinuma S, Saga K, Nimura K, Shimbo T, Umegaki N, Katayama I, Miyazaki J, Takeda J, McGrath JA, Uitto J, Kaneda Y. PDGFRalpha-positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. *Proc Natl Acad Sci USA* 2011; **108**: 6609-6614 [PMID: 21464317 DOI: 10.1073/pnas.1016753108]

37 **Lawrence WT**. Physiology of the acute wound. *Clin Plast Surg* 1998; **25**: 321-340 [PMID: 9696896]

38 **Fu S**, Liesveld J. Mobilization of hematopoietic stem cells. *Blood Rev* 2000; **14**: 205-218 [PMID: 11124108 DOI: 10.1054/blre.2000.0138]

39 **Kucia M**, Ratajczak J, Reca R, Janowska-Wieczorek A, Ratajczak MZ. Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. *Blood Cells Mol Dis* 2004; **32**: 52-57 [PMID: 14757413 DOI: 10.1016/j.bcmd.2003.09.025]

40 **Nemeth K**, Wilson T, Rada B, Parmelee A, Mayer B, Buzas E, Falus A, Key S, Masszi T, Karpati S, Mezey E. Characterization and function of histamine receptors in human bone marrow stromal cells. *Stem Cells* 2012; **30**: 222-231 [PMID: 22045589 DOI: 10.1002/stem.771]

41 **Velnar T**, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res* 2009; **37**: 1528-1542 [PMID: 19930861 DOI: 10.1177/147323000903700531]

42 **Diegelmann RF**, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 2004; **9**: 283-289 [PMID: 14766366 DOI: 10.2741/1184]

43 **Witte MB**, Barbul A. General principles of wound healing. *Surg Clin North Am* 1997; **77**: 509-528 [PMID: 9194878 DOI: 10.1016/S0039-6109(05)70566-1]

44 **Goldman R**. Growth factors and chronic wound healing: past, present, and future. *Adv Skin Wound Care* 2004; **17**: 24-35 [PMID: 14752324 DOI: 10.1097/00129334-200401000-00012]

45 **Ramasastry SS**. Acute wounds. *Clin Plast Surg* 2005; **32**: 195-208 [PMID: 15814117 DOI: 10.1016/j.cps.2004.12.001]

46 **Chen L**, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008; **3**: e1886 [PMID: 18382669 DOI: 10.1371/journal.pone.0001886]

47 **Smith AN**, Willis E, Chan VT, Muffley LA, Isik FF, Gibran NS, Hocking AM. Mesenchymal stem cells induce dermal fibroblast responses to injury. *Exp Cell Res* 2010; **316**: 48-54 [PMID: 19666021 DOI: 10.1016/j.yexcr.2009.08.001]

48 **Hocking AM**, Gibran NS. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp Cell Res* 2010; **316**: 2213-2219 [PMID: 20471978 DOI: 10.1016/j.yexcr.2010.05.009]

49 **Tasso R**, Augello A, Boccardo S, Salvi S, Caridà M, Postiglione F, Fais F, Truini M, Cancedda R, Pennesi G. Recruitment of a host's osteoprogenitor cells using exogenous mesenchymal stem cells seeded on porous ceramic. *Tissue Eng Part A* 2009; **15**: 2203-2212 [PMID: 19265473 DOI: 10.1089/ten.tea.2008.0269]

50 **Servold SA**. Growth factor impact on wound healing. *Clin Podiatr Med Surg* 1991; **8**: 937-953 [PMID: 1933739]

51 **Baum CL**, Arpey CJ. Normal cutaneous wound healing: clinical correlation with cellular and molecular events. *Dermatol Surg* 2005; **31**: 674-686; discussion 686 [PMID: 15996419 DOI: 10.1097/00042728-200506000-00011]

52 **Kim J**, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol* 2009; **37**: 1445-1453 [PMID: 19772890 DOI: 10.1016/j.exphem.2009.09.004]

53 **Mauney J**, Olsen BR, Volloch V. Matrix remodeling as stem cell recruitment event: a novel in vitro model for homing of human bone marrow stromal cells to the site of injury shows crucial role of extracellular collagen matrix. *Matrix Biol* 2010; **29**: 657-663 [PMID: 20828613 DOI: 10.1016/j.matbio.2010.08.008]

54 **Arany PR**, Cho A, Hunt TD, Sidhu G, Shin K, Hahm E, Huang GX, Weaver J, Chen AC, Padwa BL, Hamblin MR, Barcellos-Hoff MH, Kulkarni AB, J Mooney D. Photoactivation of endogenous latent transforming growth factor-β1 directs dental stem cell differentiation for regeneration. *Sci Transl Med* 2014; **6**: 238ra69 [PMID: 24871130 DOI: 10.1126/scitranslmed.3008234]

55 **Greenhalgh DG**. The role of apoptosis in wound healing. *Int J Biochem Cell Biol* 1998; **30**: 1019-1030 [PMID: 9785465 DOI: 10.1016/S1357-2725(98)00058-2]

56 **Maguire G**, Friedman P, McCarthy D, Friedman R, Maniotis A. Stem cell released molecules and exosomes in tissue engineering. *Procedia Engineering* 2013; **59**: 270-278 [DOI: 10.1016/j.proeng.2013.05.121]

57 **Ono M**, Kosaka N, Tominaga N, Yoshioka Y, Takeshita F, Takahashi RU, Yoshida M, Tsuda H, Tamura K, Ochiya T. Exosomes from

bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci Signal* 2014; **7**: ra63 [PMID: 24985346 DOI: 10.1126/scisignal.2005231]

58 **Bissell MJ**, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med* 2011; **17**: 320-329 [PMID: 21383745 DOI: 10.1038/nm.2328]

59 **Ehninger A**, Trumpp A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011; **208**: 421-428 [PMID: 21402747 DOI: 10.1084/jem.20110132]

60 **Echeverri K**, Tanaka EM. Mechanisms of muscle dedifferentiation during regeneration. *Semin Cell Dev Biol* 2002; **13**: 353-360 [PMID: 12324217 DOI: 10.1016/S1084952102000915]

61 **Grafi G**, Barak S. Stress induces cell dedifferentiation in plants. *Biochim Biophys Acta* 2015; **1849**: 378-384 [PMID: 25086338 DOI: 10.1016/j.bbagrm.2014.07.015]

62 **Chen X**, Mao Z, Liu S, Liu H, Wang X, Wu H, Wu Y, Zhao T, Fan W, Li Y, Yew DT, Kindler PM, Li L, He Q, Qian L, Wang X, Fan M. Dedifferentiation of adult human myoblasts induced by ciliary neurotrophic factor in vitro. *Mol Biol Cell* 2005; **16**: 3140-3151 [PMID: 15843428 DOI: 10.1091/mbc.E05-03-0218]

63 **Mammoto T**, Mammoto A, Ingber DE. Mechanobiology and developmental control. *Annu Rev Cell Dev Biol* 2013; **29**: 27-61 [PMID: 24099083 DOI: 10.1146/annurev-cellbio-101512-122340]

64 **Liu M**, Liu N, Zang R, Li Y, Yang ST. Engineering stem cell niches in bioreactors. *World J Stem Cells* 2013; **5**: 124-135 [PMID: 24179601 DOI: 10.4252/wjsc.v5.i4.124]

65 **Rouabhia M**, Park H, Meng S, Derbali H, Zhang Z. Electrical stimulation promotes wound healing by enhancing dermal fibroblast activity and promoting myofibroblast transdifferentiation. *PLoS One* 2013; **8**: e71660 [PMID: 23990967 DOI: 10.1371/journal.pone.0071660]

66 **Park JG**, Lee DH, Moon YS, Kim KH. Reversine increases the plasticity of lineage-committed preadipocytes to osteogenesis by inhibiting adipogenesis through induction of TGF-β pathway in vitro. *Biochem Biophys Res Commun* 2014; **446**: 30-36 [PMID: 24548409 DOI: 10.1016/j.bbrc.2014.02.036]

67 **Maniotis AJ**, Chen CS, Ingber DE. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc Natl Acad Sci USA* 1997; **94**: 849-854 [PMID: 9023345 DOI: 10.1073/pnas.94.3.849]

68 **Downing TL**, Soto J, Morez C, Houssin T, Fritz A, Yuan F, Chu J, Patel S, Schaffer DV, Li S. Biophysical regulation of epigenetic state and cell reprogramming. *Nat Mater* 2013; **12**: 1154-1162 [PMID: 24141451 DOI: 10.1038/nmat3777]

69 **Bernardino L**, Eiriz MF, Santos T, Xapelli S, Grade S, Rosa AI, Cortes L, Ferreira R, Bragança J, Agasse F, Ferreira L, Malva JO. Histamine stimulates neurogenesis in the rodent subventricular zone. *Stem Cells* 2012; **30**: 773-784 [PMID: 22893458 DOI: 10.1002/stem.1042]

70 **Panula P**, Sundvik M, Karlstedt K. Developmental roles of brain histamine. *Trends Neurosci* 2014; **37**: 159-168 [PMID: 24486025 DOI: 10.1016/j.tins.2014.01.001]

71 **Tran TD**, Zolochevska O, Figueiredo ML, Wang H, Yang LJ, Gimble JM, Yao S, Cheng H. Histamine-induced $Ca^{2+}$ signalling is mediated by TRPM4 channels in human adipose-derived stem cells. *Biochem J* 2014; **463**: 123-134 [PMID: 25001294 DOI: 10.1042/BJ20140065]

72 **Biosse-Duplan M**, Baroukh B, Dy M, de Vernejoul MC, Saffar JL. Histamine promotes osteoclastogenesis through the differential expression of histamine receptors on osteoclasts and osteoblasts. *Am J Pathol* 2009; **174**: 1426-1434 [PMID: 19264900 DOI: 10.2353/ajpath.2009.080871]

73 **Pochampally RR**, Ylostalo J, Penfornis P, Matz RR, Smith JR, Prockop DJ. Histamine receptor H1 and dermatopontin: new downstream targets of the vitamin D receptor. *J Bone Miner Res* 2007; **22**: 1338-1349 [PMID: 17547532 DOI: 10.1359/jbmr.070605]

74 **Mu X**, Peng H, Pan H, Huard J, Li Y. Study of muscle cell dedifferentiation after skeletal muscle injury of mice with a Cre-Lox system. *PLoS One* 2011; **6**: e16699 [PMID: 21304901 DOI: 10.1371/journal.pone.0016699]

75 **Téllez N**, Montanya E. Gastrin induces ductal cell dedifferentiation and β-cell neogenesis after 90% pancreatectomy. *J Endocrinol* 2014; **223**: 67-78 [PMID: 25122000 DOI: 10.1530/JOE-14-0222]

76 **Xiong XR**, Lan DL, Li J, Zi XD, Ma L, Wang Y. Cellular extract facilitates nuclear reprogramming by altering DNA methylation and pluripotency gene expression. *Cell Reprogram* 2014; **16**: 215-222 [PMID: 24738992 DOI: 10.1089/cell.2013.0078]

77 **Anokye-Danso F**, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrisey EE. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376-388 [PMID: 21474102 DOI: 10.1016/j.stem.2011.03.001]

78 **Shoshani O**, Ravid O, Massalha H, Aharonov A, Ovadya Y, Pevsner-Fischer M, Leshkowitz D, Zipori D. Cell isolation induces fate changes of bone marrow mesenchymal cells leading to loss or alternatively to acquisition of new differentiation potentials. *Stem Cells* 2014; **32**: 2008-2020 [PMID: 24715711 DOI: 10.1002/stem.1719]

79 **Nichols J**, Smith A. Naive and primed pluripotent states. *Cell Stem Cell* 2009; **4**: 487-492 [PMID: 19497275 DOI: 10.1016/j.stem.2009.05.015]

80 **Mathieu J**, Zhang Z, Nelson A, Lamba DA, Reh TA, Ware C, Ruohola-Baker H. Hypoxia induces re-entry of committed cells into pluripotency. *Stem Cells* 2013; **31**: 1737-1748 [PMID: 23765801 DOI: 10.1002/stem.1446]

81 **Booth BW**, Boulanger CA, Anderson LH, Smith GH. The normal mammary microenvironment suppresses the tumorigenic phenotype of mouse mammary tumor virus-neu-transformed mammary tumor cells. *Oncogene* 2011; **30**: 679-689 [PMID: 20890308 DOI: 10.1038/onc.2010.439]

82 **Comes S**, Gagliardi M, Laprano N, Fico A, Cimmino A, Palamidessi A, De Cesare D, De Falco S, Angelini C, Scita G, Patriarca EJ, Matarazzo MR, Minchiotti G. L-Proline induces a mesenchymal-like invasive program in embryonic stem cells by remodeling H3K9 and H3K36 methylation. *Stem Cell Reports* 2013; **1**: 307-321 [PMID: 24319666 DOI: 10.1016/j.stemcr.2013.09.001]

83 **Mead C.** The Nature of Light: What are "Photons"? *Proc SPIE* 2013; **8832**: 883202 [DOI: 10.1117/12.2046381]

84 Mead C, Ismail M. Analog VLSI and Neural Systems. Boston, MA: Springer Science & Business Media, 1989 [DOI: 10.1007/978-1-4613-1639-8]

85 **Merolla PA**, Arthur JV, Alvarez-Icaza R, Cassidy AS, Sawada J, Akopyan F, Jackson BL, Imam N, Guo C, Nakamura Y, Brezzo B, Vo I, Esser SK, Appuswamy R, Taba B, Amir A, Flickner MD, Risk WP, Manohar R, Modha DS. Artificial brains. A million spiking-neuron integrated circuit with a scalable communication network and interface. *Science* 2014; **345**: 668-673 [PMID: 25104385 DOI: 10.1126/science.1254642]

86 **Zasloff M**. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 1987; **84**: 5449-5453 [PMID: 3299384 DOI: 10.1073/pnas.84.15.5449]

87 **Koch R.** Über Desinfection. Mittheil. des kaiserl. *Gesundheitsamtes* 1881; **1**: 234-282

88 **Thormar H,** Hilmarsson H, Bergsson G. Antimicrobial lipids: Role in innate immunity and potential use in prevention and treatment of infections. In: Microbial pathogens and strategies for combating them: science, technology and education. Méndez-Vilas A, editor. *Formatex* 2013; **3**: 1474-1488

89 **Moore KS**, Wehrli S, Roder H, Rogers M, Forrest JN, McCrimmon D, Zasloff M. Squalamine: an aminosterol antibiotic from the shark. *Proc Natl Acad Sci USA* 1993; **90**: 1354-1358 [PMID: 8433993 DOI: 10.1073/pnas.90.4.1354]

90 **Lu P**, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 2012; **196**: 395-406 [PMID: 22351925 DOI: 10.1083/jcb.201102147]

91 **Liou GY**, Döppler H, Necela B, Edenfield B, Zhang L, Dawson

DW, Storz P. Mutant KRAS-induced expression of ICAM-1 in pancreatic acinar cells causes attraction of macrophages to expedite the formation of precancerous lesions. *Cancer Discov* 2015; **5**: 52-63 [PMID: 25361845 DOI: 10.1158/2159-8290.CD-14-0474]

92 **Maguire G**. Maturing from embryonic to adult policy on stem cell therapeutics. *ACS Med Chem Lett* 2014; **5**: 1264-1265 [PMID: 25516780 DOI: 10.1021/ml500396z]

93 **Williams G**, Zentar MP, Gajendra S, Sonego M, Doherty P, Lalli G. Transcriptional basis for the inhibition of neural stem cell proliferation and migration by the TGFβ-family member GDF11. *PLoS One* 2013; **8**: e78478 [PMID: 24244313 DOI: 10.1371/journal.pone.0078478]

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MINIREVIEWS

# Ovarian stem cells: From basic to clinical applications

Ozlem Bingol Ozakpinar, Anne-Marie Maurer, Derya Ozsavci

Ozlem Bingol Ozakpinar, Derya Ozsavci, Department of Biochemistry, School of Pharmacy, Marmara University, 34668 Istanbul, Turkey
Anne-Marie Maurer, Department of Hematology, School of Medicine, Marmara University, Pendik, 34668 Istanbul, Turkey
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Correspondence to: Ozlem Bingol Ozakpinar, PhD, Department of Biochemistry, School of Pharmacy, Marmara University, Tıbbiye Cad. No:49 Haydarpaşa, 34668 Istanbul, Turkey. ozlem.bingol@marmara.edu.tr
Telephone: +90-216-4142962
Fax: +90-216-3452952
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## Abstract

The field of reproductive biology has undergone significant developments in the last decade. The notion that there is a fixed reserve pool of oocytes before birth was established by Zuckerman in 1951. However, in 2004, an article published in nature challenged this central dogma of mammalian reproductive biology. Tilly's group reported the existence of ovarian germline stem cells (GSCs) in postnatal ovaries of mice and suggested that the bone marrow could be an extragonadal source of ovarian GSCs. These findings were strongly criticized; however, several independent groups have since successfully isolated and characterized ovarian GSCs in postnatal mice. The ovarian GSCs are located in the ovarian surface epithelium and express markers of undifferentiated GSCs. When transplanted into mouse ovaries, mouse ovarian GSCs could differentiate and produce embryos and offspring. Similarly, in a recent study, ovarian GSCs were found to be present in the ovaries of women of reproductive age. Conversely, there is increasing evidence that stem cells responsible for maintaining a healthy state in normal tissue may be a source of some cancers, including ovarian cancer. Cancer stem cells (CSCs) have been found in many tissues, including ovaries. Some researchers have suggested that ovarian cancer may be a result of the transformation and dysfunction of ovarian GSCs with self-renewal properties. Drug resistant and metastasis-generating CSCs are responsible for many important problems affecting ovarian cancer patients. Therefore, the identification of CSCs will provide opportunities for the development of new therapeutic strategies for treatments for infertility and ovarian cancer. In this article, we summarize the current understanding of ovarian GSCs in adult mammals, and we also discuss whether there is a relationship between GSCs and CSCs.

**Key words:** Cancer; Cancer stem cell; Germline stem cell; Ovary; Reproductivity

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**Core tip:** This review provides an overview on postnatal ovarian germinal stem cells (GSC) of mammals. The characteristics of these cells and the last developments in the field of oogenesis have been presented. We also discuss the relationship between ovarian GSCs and ovarian cancer stem cells. The identification and characterization of these two types of cells are essential for a better understanding of tumor initiation, progression and treatment.

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## INTRODUCTION

The field of the reproductive biology was revolutionized ten years ago when Johnson *et al*[1] published a study in *Nature* that challenged the long-held dogma established by Zuckerman[2] in 1951. Since that time, it has been generally believed that the ovaries of mammals do not possess renewable stem cells but instead contain a finite reserve of oocytes that diminishes through postnatal life. Although the existence of ovarian germline stem cells (GSCs) has been obviously demonstrated and fully accepted for adult females of non-mammalian species[3-5] and for adult males of a majority of species[6], the existence of ovarian GSCs in adult female mammals is still a subject of intense debate. In 2004, Johnson *et al*[1] demonstrated the existence of proliferative GSCs in the ovaries of adult mice, and recently, ovarian GSCs have been isolated and characterized in the ovaries of postnatal mice and reproductive-age women[7].

Amid the controversy created by group by Johnson *et al*[1], the presence of cancer stem cells (CSC) in ovarian cancer was established by Bapat *et al*[8], and accumulating data have provided substantial evidence for the involvement of CSCs in ovarian cancer[9-13]. Ovarian cancer (OC) is associated with enhanced tumor aggressiveness and metastasis, as well as drug resistance. The heterogeneous populations of cancer cells within an ovarian tumor tend to be more resistant to chemotherapeutic agents. In this context, the identification and characterization of CSCs in ovarian cancer is essential for a better understanding of the signaling pathways involved in tumor development and progression. In this review, we will focus on the latest developments in the field of oogenesis in the postnatal mammalian ovary. We will also discuss whether there is a link between ovarian GSCs and CSCs.

## OVARIAN GSCS IN ADULT MAMMALS

### *Existence of ovarian GSCs*

In 2004, Johnson *et al*[1] published a study that challenged the dogma established by Zuckerman[2] in 1951. The authors demonstrated that ovarian GSCs are present in the adult mouse ovary, contrary to the principle established more than 60 years ago (Table 1). In a first series of studies, Johnson *et al*[1] counted the numbers of healthy (non-atretic) and degenerating (atretic) follicles in ovaries of mice to study germ cell dynamics in female mammals. The numbers of non-atretic quiescent (primordial) and early growing (primary, preantal) follicles in ovary was higher than expected and their rate of clearance in the immature ovary (day 1-day 4) was less than expected. According to their experiments on the clearance of degenerative oocytes contained within immature follicles, from 1% to 33% of the immature follicle pool was atretic at any given time. The authors considered that the degeneration of this cell would deplete the primordial follicle reserve by young adulthood and that ovarian GSCs represent the source of oocytes produced *de novo*[1].

By using unbiased assumption-free stereological methods to count follicles in the mouse ovary, Kerr *et al*[14] demonstrated the presence of actively dividing surface epithelial cells in prepubertal mouse ovaries and supported the concept of follicle renewal in postnatal and adult ovaries in mice. This study, published in 2006, confirmed the data from the Johnson *et al*[1]'s group (Table 1).

Johnson *et al*[1] reported the presence of large ovoid cells in the surface epithelium of juvenile and young adult mouse ovaries by histological analysis. These cells seemed similar to the germline cells of fetal mouse ovaries. Immunohistochemical staining for Mouse Vasa Homologue (MVH, also called Deadbox 4 or Ddx4), a gene expressed exclusively in the germ cells in both vertebrate and invertebrate species[15], demonstrated that these large cells were of a germline lineage. Incorporation of 5-bromodeoxyuridine (BrdU) into the DNA of the MVH-positive cells demonstrated the proliferative capacity of these double-positive cells, which were localized to the ovarian surface epithelium. Moreover, postnatal ovarian expression of genes involved in the initiation of meiosis was determined by RT-PCR. Protein synaptonemal complex protein SCP3, the endonuclease Spo11, and the recombinase Dmc1 (required for the initiation of meiosis in mammals) were all detected at the mRNA level[1]. These results confirmed the presence of proliferative germ cells in postnatal mouse ovaries. Supplemental evidence of continuous folliculogenesis during post natal life was provided by grafting experiments. Ovarian fragments from wild-type mice were grafted onto hemi-ovaries of transgenic mice with ubiquitous expression of green fluorescent protein (GFP). After 3-4 wk, follicles containing GFP-positive oocytes were detected in the wild-type ovarian fragments surrounded by unlabeled granulosa cells. These results suggested that transgenic germ cells had migrated into the grafted ovarian fragment and formed new follicles in adult mice. Collectively, these findings demonstrated that proliferative GSCs exist in the postnatal mammalian ovary. The GSCs support oocyte and follicle production in the postnatal mammalian ovary. This new concept challenged the dogma of a fixed reserve of oocytes.

Following the publication of this landmark article, Johnson *et al*[1] were confronted by criticism and skepticism[16-20]. The main criticisms were related to the subjectivity of the scoring, the tissue fixation protocol, the mathematical model used to calculate the follicular dynamics, the toxicity of busulfan to follicles, the capacity of BrdU-MVH-double-positive cells for oocyte

**Table 1 Articles in the field of ovarian germline stem cells and oogenesis**

| Ref. | Main findings |
|---|---|
| Johnson *et al*[1] | Ovarian GSCs are observed within the ovarian surface epithelium and provide the adult mouse ovary with oocytes |
| Johnson *et al*[25] | GSCs are detected in mouse bone marrow and provide the postnatal mouse ovary with oocytes |
| Kerr *et al*[14] | The follicle numbers remain constant in ovaries of juvenile and early adult mice. Follicle renewal in postnatal and adult mouse ovaries is suggested |
| Zou *et al*[29] | Mouse ovarian GCS are isolated by immunoselection and characterized. Ovarian GCSs transplanted into ovaries of infertile mice undergo oogenesis and produce offspring |
| Pacchiarotti *et al*[30] | The adult mouse ovarian GSCs are isolated by FACS. Long-term expanded mouse ovarian GSCs maintain their characteristics, telomerase activity and express germ cell and stem cell markers |
| White *et al*[7] | The ovarian GSCs are isolated by FACS. Xenotransplantation of human GCSs into NOD-SCID mice leads to the formation of follicles containing oocytes |
| Zhang *et al*[32] | No mitotically active female GSCs exist in postnatal mouse ovaries |
| Lei *et al*[33] | The adult female mouse ovary does not contain active ovarian GSCs. The number of follicles produced during fetal development is sufficient to provide ovaries with oocyte in adult life |
| Park *et al*[39] | Existence of mitotically active germ cells in the postnatal mouse ovary demonstrated by a genetic approach coupled with a GSCs selection strategy |

GSCs: Germline stem cells; FACS: Fluorescence-activated cell sorting.

renewal, and the migration of GSCs to form new follicles in wild-type ovaries in the grafting studies.

Several groups criticized the methods used by Johnson *et al*[1]'s group. Moreover, some of them proposed alternative explanations for Johnson's findings. First, the numbers of preantral and antral immature follicles reported as atretic by the Johnson *et al*[1]'s group group were very high and in contrast to previous studies[16]. Scoring atretic follicles represents a subjective and not reliable method for estimating the rates of follicular atresia[16,17]. Moreover, the morphological appearance of the follicles can be modified by the use of a harsh fixative[16,17]. Consequently, healthy and atretic immature follicles may have been misclassified in Johnson *et al*'s study, leading to an overestimation of the number of atretic immature follicles[17]. The mathematical model of the dynamics of follicle progression was also questioned by several authors. Johnson *et al*[1] were criticized for applying the rate of follicle disappearance from the CBA/Ca mouse strain, reported as a fast-depleting strain, in comparison to the C57Bl/6 strain[16]. To verify Johnson *et al*[1]'s results, Bristol-Gould *et al*[18] proposed examining whether the initial population of follicles was sufficient to support fertility in adulthood by using a mathematical model of the dynamics of follicle progression. These authors counted the follicles in each stage from postnatal day 6 through to 12 mo in mice. The dynamics of the follicle population were simulated according to two distinct models: a fixed pool model and a stem cell model in the mouse ovary. The fixed pool model accurately reflected the experimental decrease in follicle numbers and allowed the authors to refute the concept of GSC replenishment in the adult ovary[18]. Johnson's assumption that busulfan was only toxic to GSCs and does not kill primordial follicles was questioned by two groups[16,17]. The loss of primordial follicles after busulfan treatment was considered to be a consequence of busulfan toxicity to primordial follicles and not due to the depletion of GSCs[17]. The evidence that BrdU and MVH double-positive cells in the epithelial layer of juvenile and young adult mouse ovaries represented functional GSCs was also challenged[16-20]. The critics of Johnson *et al*[1]'s group findings suggested some alternative explanations for the detection of these cells in the ovarian surface epithelium. They asserted that these putative GSCs could be germ cells migrating out of the ovary, as had been reported for these cells in the past[16,21]. Alternatively, these double-labeled cells could be oocytes from primordial follicles, released by the ovary[16,20]. The grafting experiments were also subject to reinterpretation by opponents of the findings from the Johnson *et al*[1]'s group[16,20]. The plasticity of the mouse ovary has recently been demonstrated in experiments with chimeric ovaries[22]. In addition, the mouse ovarian tissue has been shown to reaggregate after injury[23]. The chimerism observed in the wild-type transplanted ovaries could result from the subsequent ovarian repair after the tissue trauma due to the transplantation rather than the generation of follicles *de novo*[16,20].

In response to the critics, in a new study, Tilly and his group counted the numbers of atretic and non-atretic follicles in wild-type (WT) female mice and caspase-6 knockout female mice[24]. They observed that the number of primordial follicles in caspase-6 deficient young adult females was nearly double the number of primordial follicles in WT females. As no decrease in the number of atretic follicles was detected, the authors interpreted the increase in the primordial follicle pool between birth and adulthood in caspase-6 mutant females as a consequence of neo-folliculogenesis.

Following this study, Skaznik-Wikiel *et al*[1]'s reinforced the validity of their findings and the concept of postnatal oocyte and follicle production in mammals[24].

### *Bone marrow as a source of GSCs*

In 2005, Johnson *et al*[25] proposed a new concept that further challenged the dogma of a fixed reserve of oocytes in the ovaries of postnatal females. They

suggested that the bone marrow (BM) could be a source of germ cells for the postnatal mouse ovary. The authors demonstrated that BM cells isolated from adult female mice expressed octamer-binding transcription factor-4 (Oct-4), MVH, deleted in azoospermia-like, a germ cell-specific RNA-binding protein (Dazl), Stella (a maternal factor) and Fragilis (Ifitm3, interferon-induced transmembrane protein 3), which are specific germline markers in mice. Transplantation of BM from adult wild-type female mice was performed with adult female mice treated with cyclophosphamide and busulfan to destroy the existing germ cell pools, and also into ataxia telangiectasia-mutated (Atm)-deficient female mice, which are unable to produce mature cells. After transplantation, oocyte-containing follicles at all stages of maturational development were detected in the ovaries of the chemotherapy-sterilized wild-type and Atm-mutant recipient mice. Moreover, oocytes and follicles were detected in the ovaries more than 11 mo after the initial BM transplantation. Johnson *et al*[25] and his group concluded that the putative BM-derived germline cells were able to support long-term oocyte production.

Consequently, they hypothesized that the BM-derived germ cells could be transported by the peripheral blood (PB) to replenish the ovaries. Transplantation of PB mononuclear cells from transgenic female mice with GFP expression driven by an Oct-4 promoter was performed with chemotherapy-sterilized wild-type and atm-mutant female mice. GFP-positive oocytes were observed in the ovaries of the chemoablated adult wild-type and atm-mutant female mice. Therefore, PB was capable of generating oocytes. GSCs derived from the BM could circulate in the PB to colonize the ovaries. Johnson *et al*[25] and his group concluded that the BM could be considered as a potential source of germ cells that could support oocyte production in adulthood.

The criticism of this new concept developed by Johnson *et al*[25]'s group was again severe[26,27]. A group of 16 authors published a short correspondence in which they questioned the Johnson *et al*[25] group's findings. Their main criticisms concerned the expression of germ cells markers on BM and PB cells, the lack of a reciprocal experiment showing that mononuclear PB cells from wild-type mice could produce oocytes in Atm-Oct4/GFP mutant mouse ovaries, the capacity of generated oocytes to sustain fertilization and subsequent embryo development, and the recurrent question of ovarian failure in women treated by chemotherapy and subsequent BM or PB transplantation.

Subsequently, the Johnson *et al*[28] group published documented responses to the critics to clarify the misinterpretations of their studies. Among the different points discussed by Telfer *et al*[26], they defended the germline specificity of the markers used in their studies. They also clearly explained that GFP could not have been absorbed by oocytes from the blood because blood cells from GFP-transgenic males did not induce the generation of GFP-expressing oocytes in wild-type female recipients. The authors reported some cases of fertility restoration in women treated with high-dose chemotherapy followed by BM or PB transplantation. They also suggested that the BM-derived stem cells involved in blood cell regeneration might be different from those that are able to promote de novo oogenesis, noting that the protocols are designed for the recovery of hematopoiesis and not oogenesis.

After the Johnson *et al*[25] publication, Eggan *et al*[27] performed parabiosis experiments with adult female mice to test the physiological relevance of BM-derived germ cells to contribute to oogenesis. To evaluate the capacity of circulating BM-derived GSCs to colonize ovaries, they examined ovulated oocytes from wild-type and ubiquitously-expressing GFP female mice that were surgically joined in parabiotic pairs at the age of 4-8 wk. The parabiotic pairs showed a high level peripheral blood chimerism after 6-8 mo (65% GFP-positive blood leukocytes in both mice).

After superovulation, no chimerism of the oocytes was observed in the parabiotic mice. Moreover, occasional GFP-positive cells associated with wild-type oocytes in the cumulus masses were positive for CD45, a pan-hematopoietic marker, demonstrating that these cells were circulating blood cells. The authors concluded that although these circulating cells could associate with ovulated oocytes in the ovary, they were not involved in the production of oocytes; the authors suggested that these hematopoietic cells may play an immune role in ovaries.

In subsequent studies, the investigators treated wild-type mice with cyclophosphamide and busulfan 1 d before joining them to GFP-expressing partners for a duration of 2 wk or 2 mo. In the treated and untreated control parabionts, PB and BM exhibited extensive leukocyte chimerism whereas no chimerism was observed in the oocytes. As indicated above, it was also shown that all GFP-positive cells associated with the ovulated cells were CD45 positive in both the treated and treated wild-type parabiotic mice. In addition, 2 mo after the chemoablative treatment, the ovaries still contained oocytes, revealing that the chemotherapy did not induce a complete depletion of oocytes in the treated parabionts.

The authors then suggested that a direct intravenous transplantation of bone marrow cells might introduce cells into the circulation that might be able to contribute to oogenesis. To investigate this possibility, wild-type mice were treated by chemotherapy (cyclophosphamide and busulfan) or sterilized by low-dose total body irradiation. The mice were injected or transplanted with GFP-transgenic BM cells and were superovulated after 2 mo. Chimerism was observed in hematopoietic stem cells in both chemotherapy-treated and irradiated recipient mice. However, no GFP-positive oocytes were detected. Furthermore, a small number of oocytes was ovulated in chemotherapy-treated mice. No oocytes were detected in irradiated mice.

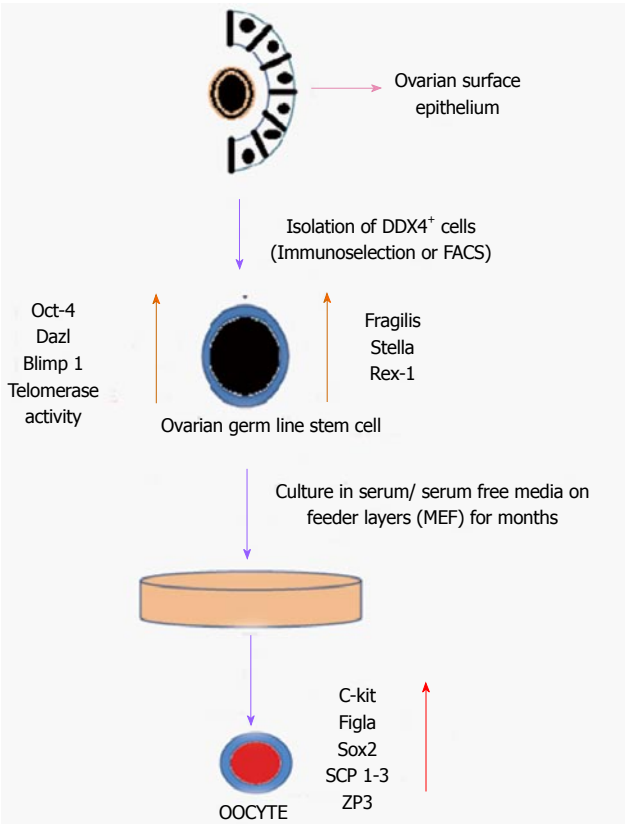


**Figure 1 Schematic diagram of isolation and differantiation of ovarian germ stem cells.** DDX4: DEAD box polypeptide 4; FACS: Fluorescence activated cell sorting; MEF: Mouse embryonic fibroblast; SCP 1-3: Synaptonemal complex protein 1,2,3; ZP3: Zona pellucida protein 3.

In conclusion, these findings demonstrated that PB and BM cells did not directly contribute to the formation of ovulated oocytes or their recovery after chemotherapy. Additionally, the BM-derived cells combined with oocytes belonged to the hematopoietic lineage, and oocytes in the adult mammalian ovaries could not be regenerated by circulating germ cell progenitors.

### *Isolation and characterization of GSCs from mouse and human ovarian tissues*

Despite the controversies, the existence of GSCs was finally reported in 2009 by Zou *et al*[29]. They developed a strategy to isolate GSCs from neonatal and young adult mouse ovaries by immunoselection with the antigen MVH (Figure 1). The isolated MVH-positive cells were morphologically similar to type A spermatogonial cells (SSCs). They possessed large cell bodies with little cytoplasm and large nuclei. These MVH-positive cells were present in the ovarian surface epithelium. The isolated MVH-positive cells were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) cell feeders. The MVH-positive cells incorporated BrdU in culture, and the BrdU-MVH double-positive cells were thus considered to be ovarian GSCs. After several passages, the MVH-positive cells formed compact clusters with blurred cell boundaries that did not resemble embryonic stem (ES) cell colonies. The nGSCs from neonatal mice could be maintained in culture for more than 15 mo (68 passages) and maintained a morphology similar to that of freshly isolated nGSCs. GSC from adult (aGSCs) mice had been passaged in culture for more than 6 mo (more than 25 times). The cultured nGSCs and aGSCs expressed Oct-4, MVH, Dazl, Blimp-1 (a transcriptional repressor), Fragilis, Stella, and Rex-1 (zfp-42, zinc-finger protein 42), which are markers of undifferentiated GSCs (Figure 1). The cells did not express c-Kit (stem cell factor receptor), Figla (a meiosis-specific marker), Sox-2 (a key transcription factor that regulates stemness), Nanog (a pluripotency sustaining factor), synaptonemal complex protein 1,2,3 (Scp1-3), or zona pellucida protein 3 (ZP3), which are established markers of differentiated oocytes. This expression profile revealed that the cultured cells possessed undifferentiated GSC phenotypes. Moreover, the cultured cells showed high telomerase activity. After the transplantation of GFP-expressing GSCs into the ovaries of chemotherapy-ablated wild-type mice, the ovaries contained many GFP-positive oocytes at all stages of development. Moreover, the transplanted mice produced offspring after mating with a wild-type male. Eighty-two percent of nGSCs and 80% of aGSCs produced F1 offspring. The offspring were fertile and could also generate F2 GFP-transgenic progeny (Figure 1).

Furthermore, Pacchiarotti *et al*[30] used fluorescence-activated cell sorting (FACS) to isolate GSCs from the ovaries of a transgenic mouse model. In this model, GFP was expressed under the control of the Oct-4 promoter. The GFP-Oct-4-positive isolated cells were expanded on mouse MEFs for more than 1 year. GFP-Oct-4-positive cells formed round, flat colonies. These colonies maintained their telomerase activity and normal karyotype after 20 passages. The cells expressed germ cell and stem cell markers, such as germ cell nuclear antigen, c-kit, Oct-4, Nanog and GFR-$\alpha$1 (the receptor for GDNF), and they possessed a normal karyotype. In the presence of growth factors, these GSC cells formed embryoid bodies (EB) and expressed specific markers for the three germ layers. In the absence of growth factors, EBs produced oocyte-like cells. The authors' results thereby indicated the existence of GSCs in the postnatal mouse ovary.

In a recent study published in Nature Medicine, White *et al*[7] reported the isolation and characterization of ovarian germ stem cells from mouse and human ovarian tissue. GSCs were isolated and purified by FACS using the Ddx4 protein. Isolated mouse and human OSCs express high levels of Prdm1 (PR domain containing 1 with ZNF domain, referred to as Blimp1), Dppa3 (developmental pluripotency-associated 3, referred to as Stella), and Ifitm3, which identify primitive germ cell lines in mammals. Moreover, mouse

and human OSCs express high levels of the catalytic subunit of telomerase (Tert), a feature of pluripotent stem cells and germ cells. The Ddx4-positive mouse and human ovarian GSCs were maintained on MEF-free cultures. After 10-12 and 4-8 wk of culture respectively, the mouse and human ovarian GSCs formed actively dividing germ cell colonies. Gene expression analysis of cultured cells confirmed the maintenance of early germ line markers. Moreover, mouse and human ovarian GSC cells cultured *in vitro* spontaneously underwent oogenesis 24-48 and 72 h after each passage, respectively. Ploidy analysis of the cultured mouse and human GSCs detected 4n, 2n, and 1n populations of cells. These 1n cells were purported to be haploid germ cells.

Transplantation of GFP-expressing mouse ovarian GSCs into ovaries of non-chemotherapy-conditioned wild-type mice resulted in the formation of developing follicles containing GFP-positive oocytes. *In vitro* fertilization of the GFP-expressing oocytes led to the formation of embryos expressing GFP. In co-cultures of human GFP-transduced ovarian GSCs with adult ovarian cortical tissue, GFP-positive oocytes were found to be enclosed by GFP-negative somatic granulosa cells; these were present in tightly compact structures that resembled follicles within 24 h after seeding. GFP-expressing human GSCs injected into fragments of human adult ovarian cortical tissues were also transplanted into NOD-SCID female mice. Human ovarian grafts contained primordial and primary follicles with GFP-negative oocytes and also immature follicles containing GFP-positive oocytes.

After the publication of this new study, Oatley and Hunt published a brief commentary to raise some questions regarding these latest findings[31]. They questioned whether the purported GSCs represented true stem cells. Oatley and Hunt also questioned the increase in haploid cells, indicating that oocytes completed both meiotic divisions *in vitro* and proposed that the oocytes underwent an abnormal maturation that does not occur *in vivo*. They also criticized the lack of studies on the normalcy of embryos derived from GFP-positive GSCs, arguing that the developmental potential and the genetic quality of the embryos needed to be tested.

### *Latest developments in the area of GSCs*

After the publication of the above-mentioned studies, two different groups presented contradictory results[32,33]. These investigators refuted the existence of GSCs in the adult ovary and provided evidence supporting the dogma of a fixed pool of oocytes after birth.

In 2012, Zhang *et al*[32] claimed that no mitotically active female germline progenitors existed in postnatal mouse ovaries. In their study, they challenged the existence of ovarian GSCs using a genetic approach. They developed a multiple fluorescent Rosa26rbw/+;Ddx4-Cre germline reporter mouse model to trace the development of Ddx4-expressing ovarian cells *in vitro* and *in vivo*. This model expresses GFP ubiquitously. The Ddx4-expressing germline cells and the non-expressing Ddx4 somatic cells can be recognized by a difference in color. The authors tested the ability of adult mouse ovaries to support the formation of new follicles when provided with female primordial germ cells. Transplantation of enhanced GFP (EGFP)-expressing fetal ovarian cells into adult wild-type female mice led to the formation of fluorescent follicles at different stages of development in the cortex and the medulla of the recipient ovaries. Consequently, the adult mouse ovaries supported *de novo* follicular formation if progenitor cells that were able to differentiate into oocytes were provided. In addition, the adult mouse ovary cells did not transform into oocytes or granulosa cells, and no chimeric follicles were observed. Moreover, when the EGFP-expressing fetal ovarian cells were transplanted into the ovaries of chemotherapy-sterilized WT adult female mice, only newly formed EGFP-positive follicles were detected in the ovaries, which suggests that postnatal mouse ovaries are able to sustain *de novo* follicle formation when provided with exogenous germline progenitors.

The ovaries of Rosa26rbw/+;Ddx4 Cre postnatal mice were dissociated, and single cells were cultured to determine the proliferation capacity of Dxd4-expressing cells (Red FP positive cells, RFP).

None of the RFP-positive ovarian cells underwent any cell divisions, and they rapidly died off. In long-term cultures on feeders, RFP-positive ovarian cells did not form any colonies. Some Dxd-4-negative ovarian cells formed colonies of cells that were passaged stably, but the cells did not express germ cell markers. These cells did not participate in follicle formation after injection into adult wild-type ovaries. The authors concluded that these clonal cells presented a stem cell-like morphology, but they were not functional female germline progenitors.

Recently, another group published a study supporting the dogma of a fixed pool of oocytes after birth[33]. By using a sensitive lineage-labeling system created in transgenic mice (Rosa26-YFP mice), Lei and Spradling affirmed that the primordial follicles generated during fetal life in mice are sufficient to support adult oogenesis. Moreover, female mouse ovaries do not contain active GSCs, and they do not produce new oocytes *in vivo*[33].

In 2013, Woods *et al*[34] and his group, published a review on ovarian GSCs that took into consideration two commentaries[31,35] released after the publication of the studies by White *et al*[7] and by Zhang *et al*[32]. Woods *et al*[34] responded to the comments, analyzed the findings of the Zhang *et al*[32] study, and, more importantly, defended their point of view on the existence of ovarian GSCs and oogenesis in postnatal life in mammals.

Woods *et al*[34] considered their strategy for the purification of mammalian GSCs from the postnatal ovary to be a validated and reliable method. The protocol using an antibody against the extracellular epitope of the Ddx4 protein exposed on the surface of the plasma membrane of GSCs was also confirmed to isolate these cells from oocytes, which possess only cytoplasmic Ddx4. To respond to Oatley *et al*[31] about their doubts about the validity of the stem cell properties of the ovarian GSCs, Woods *et al*[34] explained that the GSCs expressed the same markers as primitive germ cells before and after culture, as described in their publication[7]. In cultures lacking somatic granulosa cells, GSCs-derived oocytes can exit meiotic arrest and complete both meiotic divisions; thereafter, they exhibit a haploid status, similar to spontaneous oocyte activation *in vivo* when the regulation by somatic granulosa cells is disrupted by ovulation[36]. With the support of data obtained from several groups[29,30] Woods *et al*[34] claimed that GSCs have the capacity to differentiate into fully mature eggs that can be fertilized to generate normal and viable embryos and offspring in response to the criticisms expressed by Oatley and Hunt.

In the second part of their article, Woods *et al*[34] and his coauthors also questioned the results reported by Zhang *et al*'s group[32]. They affirmed that the transgenic mouse model used by Zhang *et al*[32] possessed a permanent genetic alteration in cells that have activated the *Ddx4* gene promotor region. They stated that the fluorescent recombinant reporter gene would be expressed even if the endogenous *Ddx4* gene is not active anymore. Zhang *et al*[32] did not isolate GSCs from the ovaries of their recombinant mice using the methods reported by other groups because they refuted the existence of a Ddx4 protein exposed on the extracellular surface of the plasma membrane. In experiments involving the transplantation of dissociated fetal ovarian cells into wild-type adult mice, Zhang *et al*[32] did not separate the fractions of purified germ line progenitors (primordial germ cells) and fetal follicular somatic cell progenitors to observe neofolliculogenesis in the adult ovary. A fetal somatic cell population or a fetal germ population transplanted separately could stimulate the adult ovary to recruit its own germ cell or somatic cell progenitors to sustain new follicle formation. Morphologic and genetic profiles of the RFP-positive postnatal ovarian observed in cultures were also questioned. The size of the Ddx4-expressing cells was significantly larger than that of the GSCs (5-8 mm in diameter). The method of selecting recombinant cells from mouse ovaries was discussed because Zhang *et al*[32] recovered the cells by the filtration of crude extracts of mice ovary with a size cutoff of 40 μm. Small immature oocytes present in postnatal mouse ovaries would not have been retained in the filter. The Ddx4-expressing cells observed in this study should have been considered as oocytes rather than putative GSCs, explaining their incapacity to proliferate in cultures. Identification of the Ddx4-expressing cells needed to be addressed by polymerase chain reaction (PCR)-based analysis of the gene expression patterns of GSCs and oocytes, which would have certainly given positive results for oocyte-specific genes.

Finally, in the discussion of their article, Tilly *et al*[34] compared their findings on ovarian stem cells with the observations established for the germinal stem cells in females of non-mammalian species, such as Drosophila and in males of mammalian species, such as mice. Although the reproductive period in women has a limited duration of 3 decades, Tilly argued that the presence of germinal stem cells in the ovaries should be accepted as it is in Drosophila, in which the GSCs persist after the failure of reproductive life[37], or in male mice, in which the presence of germinal spermatogonial stem cells has been detected after spermatogenic failure[38]. The authors defended the idea that mammalian females should not be excluded from that standard.

Following the puzzling questions about the Zhang *et al*[32] study, Park *et al*[39] and his group decided to carry on experiments using transgenic Ddx4-Cre mice crossed with mice carrying a Rosa26$^{tdTm/tdTm}$ fluorescent gene reporter to identify GSCs in adult mouse ovaries[39]. In experiments performed according to the protocol used in the study by Zhang *et al*[32], Park *et al*[39] confirmed that cells collected from crudely dispersed ovaries from recombined mice at postnatal day 8 and selected by filtration were immature oocytes based on PCR analysis of oocyte-specific markers. Moreover, the tdTM/Ddx4 double-positive cell population purified by FACS from dissociated Ddx4-Cre;Rosa26$^{tdTm/+}$ ovaries expressed the primitive germ cell markers Prdm1, Dppa3, Ifitm3, Tert, and Dazl but did not express the oocyte-specific markers newborn ovary homeobox (Nobox ), ZP3, and growth differentiation factor 9 (Gdf9). In addition, Ddx4 mRNA was detected in freshly purified tdTM/Ddx4 double-positive cells, although it was present at a low level. Cultures of the double-positive cells resulted fluorescent germ cell colonies that were mitotically active. In this study, Park *et al*[39] demonstrated that a genetic approach associated with a FACS cell selection based on extracellular Ddx4 was a reliable and pertinent strategy for the isolation of GSCs from the adult mouse ovary.

From the studies described above, we can infer that the presence of GSCs in the adult ovary remains a subject of intense debate and controversy, and the field of ovarian biology is undergoing profound changes. Progress in ovarian biology has also been made since the discovery of ovarian CSC in 2005[8]. The characterization of specific ovarian CSCs based on markers of stemness has also generated intense research and discussion. In the following section, we will focus on the characteristics of ovarian CSCs and their relationship with

**Table 2 Markers of ovarian cancer stem cells**

| Marker | Ref. |
|---|---|
| Side population | Szotek *et al*[9] |
| CD44/CD117 | Zhang *et al*[10] |
| Natriureticpeptide receptor A | Kong *et al*[11] |
| CD133 | Ferradina *et al*[45] |
| CD24 | Gao *et al*[13] |
| DEAD box polypeptide 4 | Hashimoto *et al*[12] |

DEAD: Asp-Glu-Ala-Asp.

GSCs.

## OVARIAN CANCER STEM CELLS: IS THERE ANY RELATIONSHIP BETWEEN GSCS AND CANCER STEM CELLS?

To date, the question concerning the cellular origin of cancer is still debated. Two major hypotheses have been proposed regarding the formation of cancer: (1) Clonal evolution, or a stochastic model; and (2) a CSC model[40]. In the first model, every cell within a tumor mass has the equivalent self-renewal capacity and multilineage potency to drive tumor development. In the CSC model, cancer originates from a small rare population of undifferentiated stem cells with self-renewal capacity. Given this theory, it is not surprising that the CSC hypothesis has gained considerable attention in recent years.

As it is known, CSCs share similar biological properties with normal somatic stem cells, including an extensive self-renewal capacity, expression of specific stem cell surface markers and genes and common signaling pathways. However, CSCs differ significantly from normal stem cells in their drug resistance and metastatic activities. Therefore, recent developments in the field of stem cell biology have led to the reconsideration of the definition of cancer. Many researchers suggest that cancer is a stem cell disease and focus on the CSCs theory to understand which cancer cells are responsible for the development of tumors, chemoresistance and high rates of recurrence.

Epithelial ovarian cancer (EOC) is the most lethal of all gynecological malignancies worldwide and the seventh leading cause of cancer-related death[41]. It is generally often diagnosed at late stages of the disease. Greater than 90% of EOCs arise from the ovarian surface epithelium (OSC). The molecular and cellular mechanisms underlying EOC remain poorly understood because of the complexity and heterogeneity of the tumor development process[42].

Accumulating experimental data have demonstrated that EOC is a stem cell-driven disease[43,44]. Over the last few years, several studies have focused on the isolation, identification, and characterization of stem cells from ovarian cancer cell lines, primary tumor tissues and ascites from cancer patients. Although no universal single marker has been found to isolate ovarian CSCs, several putative markers have been identified by several groups (Table 2). However, the isolation and characterization of CSCs have been the subject of extensive studies and the experimental strategies are still a matter of debate.

The first evidence for the existence of a CSC population in EOC patients was reported by Bapat *et al*[8] The authors isolated a single tumorigenic clone from the ascites fluid of an ovarian cancer patient and defined the characteristics of this cell clone, which was identified as a stem/progenitor cell. They suggested that ovarian cancer may be the result of transformation and dysfunction of stem cells in the ovary.

Subsequently, Szotek *et al*[9] identified stem cell-like cells, or side population (SP) cells, in the murine transgenic epithelial ovarian cancer cell line MOVCAR-7, the human ovarian cancer cell lines OVCAR 3, SKOV-3 and IGROV-1 and ascites from a small group of ovarian cancer patients using the dye efflux marker SP. Then, they went on to characterize the cell surface markers of these cells by flow cytometry. The authors found that c-kit/117 and CD44 were expressed in the mouse SP cells. However, human SP cells were not further functionally characterized in this study.

In 2008, Zhang *et al*[10] isolated and identified a self-renewing subpopulation of cancer-initiating cells from human ovarian primary tumor tissues for the first time. They showed that these cells expressed various stem cell markers, including stem cell factor, Nanog, ATP Binding Cassette G-member protein (ABCG2), and Oct-4. These cells also demonstrated chemoresistance to cancer therapeutics and were able to initiate tumors in mice, as determined by evaluating the $CD44^+/CD117^+$ phenotype. Subsequent studies have demonstrated that CD133(+) cells have higher clonogenic and proliferative potential than CD133(-) cells in primary ovarian tumors[45,46]. This result is not consistent with a recently published study indicating the CD133 expression in tumorigenic ovarian cancer cells is heterogeneous[47]. Similarly, contradictory results have been obtained using CD24 for the characterization of ovarian CSCs[13,48].

Although the origin of CSCs has been debated for a long time, a common consensus on this issue has not been reached. While some authors support the hypothesis that CSCs originate from normal stem cells undergoing malignant transformation[49,50], *in vitro* cancer models have shown that CSCs do not arise from normal stem cells[51]. Although several putative ovarian CSC markers have been identified, these markers can also be found on normal stem cells[52]. To understand the relationship between CSCs and normal stem cells, the gene expression profiles in human ovarian stem cells and ovarian cancer was analyzed, and the results showed that the expression of some genes is significantly reduced in malignant ovarian tumors but relatively unchanged in benign tumors compared to normal ovary[53]. Surprisingly, in a more recent study,

a link between normal stem cells and CSCs in ovarian cancer was established[54]. The authors analyzed the expression patterns of the ovarian GSC marker DDX4 and the ovarian CSC marker CD133 in ovarian cancers using tissue microarrays. They found that the levels of both DDX4 and CD133 were significantly increased in ovarian cancer, and the expression pattern of DDX4 was similar to that of CD133. Even more interesting, they showed that almost all CD133-positive cancer cells also expressed DDX4 whereas the CD133-negative cells did not.

The genetic program regulating the self-renewal and differentiation of CSCs plays a key role in cancer initiation, invasion and migration. A great body of evidence suggests that epigenetic changes and chromosomal alterations are responsible for tumor development including colorectal[55], breast[56], prostate[57], and hepatocellular carcinoma[58]. Epigenetic changes include altered DNA methylation, chromatin remodeling and non-coding small RNA (miRNA) expression. Recent developments in the understanding of the role of epigenetic mechanisms have shed new light on carcinogenesis. Taken together, similar to other cancers, determination of the epigenetic mechanisms involved in ovarian cancer opens promising new therapeutic avenues for treatment.

It has been emphasized that DNA methylation might play a seminal role in tumor formation, development and metastasis. DNA methylation of the cytosine residues of CG (also designated as CpG) dinucleotides is the most well-known epigenetic mechanism. Long-term silencing of genes in ES cells is under the control of the polycomb complex of proteins (PcG), and recent findings have shown that stem cell PcG targets are up to 20-fold more likely to have cancer-specific promoter methylation than nontargets, supporting a stem cell origin of cancer, including ovarian cancer[59,60]. Moreover, Baba *et al*[52] demonstrated that CD133 expression, which is a somatic stem cell marker, in ovarian cancer is directly controlled by both histone modifications and promoter methylation. Additionally, specific methylated DNA markers can be detected in the serum, plasma and peritoneal fluid of ovarian cancer patients[61].

MicroRNAs (miRNAs) are small noncoding RNAs that control gene expression post-transcriptionally and play pivotal roles in stem cell biology. Interestingly, emerging evidence suggests that some miRNAs are differentially expressed in CSCs and somatic cells and even in cancer cells, which indicates that these miRNAs may be involved in the regulation of cancer-related processes[62-65] Recently, the involvement of miRNAs in the tumorigenesis, metastasis and drug resistance of EOC has been increasingly reported; therefore, it has been proposed that miRNAs could present a novel therapeutic strategy for the management of ovarian cancer.

Guo *et al*[65] showed that miR-9 was downregulated in human ovarian cancer compared to normal ovary, and overexpression of miR-9 repressed cell growth. Li *et al*[66] examined the role of miR-27a in the development of drug resistance in ovarian cancer cells, and they found that inhibition of miR-27a decreased the expression levels of the multi drug resistance 1 (MDR1) gene and increased paclitaxel-induced apoptosis. Another recent study reported aberrant miRNA expression in ovarian cancers compared to normal ovary. Iorio *et al*[67] reported the overexpression of miR-200a, miR-141, miR-200c, and miR-200b, as well as the downregulation of miR-199a, miR-140, miR-145, and miR-125b1, in ovarian cancer cell lines.

In light of these findings, it is clear that the CSCs play an important role in the development and maintenance of EOC, and epigenetic changes are responsible for the behavior of cancer progenitor cells and their progeny.

## CONCLUSION AND FUTURE PERSPECTIVE

The field of reproductive biology is still divided over the possibility of neo-oogenesis in the mammalian adult ovary between the supporters of this new theory[1,7,29,30] and the opponents who subscribe to the long-held dogma of a fixed number of follicles at birth[32,33,68]. In addition, a recurrent question concerns the physiological role of GSCs in the adult mammalian ovary. Nonetheless, the isolation and characterization of GSCs from human ovaries open new perspectives to design protocols for producing mature, competent oocytes *in vitro* for clinical application in assisted reproduction techniques (ART).

During the past years, attempts to generate competent oocytes from ES cells and induced pluripotent stem cells have failed to meet expectations, and fertilization of ESC-derived oocytes remains to be demonstrated[69,70]. Recently, stem cells derived from newborn mouse skin have been shown to produce oocytes when cultured in an appropriate environment, but these oocytes were not able to undergo complete maturation[71]. Therefore, as a pure stem cell population that can be easily isolated, female GSCs provide a model to explore postnatal oogenesis in mammals. In addition, these cells may plausibly contribute to the preservation of sterility in women.

Many unanswered questions in the development of oocytes from GSCs remain to be addressed. Cultures of GSCs facilitate the characterization of factors that contribute to the development and regulation of oocytes[72]. Importantly, the epigenetic program and modifications involved in *de novo* oocyte formation in the adult ovary need to be further established[73]. A better understanding of these mechanisms of regulation may provide a means to stimulate the generation of oocytes in adult human ovaries *in vivo*.

The question of menopause also remains to be addressed. If ovaries can generate new oocytes during adulthood, it can therefore be suggested that the process of menopause may be the consequence of

GCS and ovarian somatic cell aging and not the result of depletion of the oocyte pool. Aging GSCs may arrest to differentiate into oocytes, and/or the environment of the GCS niche may fail to sustain oocyte generation. Therefore, the issue of menopause occurrence is not incompatible with the concept of postnatal neo-oogenesis.

Translational applications of germinal stem cell-based strategies in the field of infertility treatment in women may be achievable in the near future. Before the use of treatments that negatively impact fertility in women, samples taken from the ovaries could facilitate the isolation of GSCs for cryopreservation and/or for ex vivo expansion and subsequent differentiation into oocytes. Cultured GSCs could be transferred back into the ovaries after treatment to support neo-folliculogenesis, or GSC-derived cultured oocytes could be fertilized in a program of *in vitro* fertilization[74].

Although these potential clinical applications give hope for the future, we are a considerable way from using female GSCs in ART to treat infertility in women.

OC is the most lethal of all gynecological malignancies worldwide. To better understand cancers, researchers are now focusing on identifying the relationship between CSCs and normal stem cells. While both stem cell types have the ability to self-renew, common signaling pathways and similar gene expression profiles, these important properties make it difficult to isolate and distinguish these cells from each other. Additionally, the field faces major challenges, including the absence of specific cell surface markers, inadequate isolation and differentiation protocols, the paucity of genome-wide studies, and also ethical restrictions.

Taken together, it remains uncertain which ovarian cancer phenotype follows the stem cell model due to the complexity and heterogeneity of EOC. On the other hand, the initiation and progression of EOC is characterized by the activation of oncogenes and the switching of tumor suppressor gene expression by epigenetic mechanisms[75-77]. More importantly, there is evidence on the epigenetic regulation of pluripotency transcription factors, signaling mechanisms and self-renewal pathways that play a critical role in the transformation of GSCs into CSCs in EOC. This knowledge provides us with not only a better understanding of how cancer cells may arise from germinal stem cells, but it also contributes to the development of new technologies or strategies in the management of EOC.

Over the last several years, a great body of evidence supports the idea that ovarian CSCs are responsible for metastasis and drug resistance after chemotherapy in this life-threatening disease. The lack of markers for early detection is the main obstacle to effective treatment strategies. A better understanding of the molecular pathogenesis of EOC is needed to develop new drug therapies and/or diagnostic biomarkers.

## REFERENCES


1 **Johnson J**, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* 2004; **428**: 145-150 [PMID: 15014492 DOI: 10.1038/nature02316]

2 **Zuckerman S**. The number of oocytes in the mature ovary. *Recent Prog Horm Res* 1951; **6**: 63-108

3 **Kirilly D**, Xie T. The Drosophila ovary: an active stem cell community. *Cell Res* 2007; **17**: 15-25 [PMID: 17199109 DOI: 10.1038/sj.cr.7310123]

4 **Nakamura S**, Kobayashi K, Nishimura T, Higashijima S, Tanaka M. Identification of germline stem cells in the ovary of the teleost medaka. *Science* 2010; **328**: 1561-1563 [PMID: 20488987 DOI: 10.1126/science.1185473]

5 **White YA**, Woods DC, Wood AW. A transgenic zebrafish model of targeted oocyte ablation and de novo oogenesis. *Dev Dyn* 2011; **240**: 1929-1937 [PMID: 21761478 DOI: 10.1002/dvdy.22695]

6 **Brinster RL**. Male germline stem cells: from mice to men. *Science* 2007; **316**: 404-405 [PMID: 17446391 DOI: 10.1126/science.1137741]

7 **White YA**, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med* 2012; **18**: 413-421 [PMID: 22366948 DOI: 10.1038/nm.2669]

8 **Bapat SA**, Mali AM, Koppikar CB, Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res* 2005; **65**: 3025-3029 [PMID: 15833827 DOI: 10.1158/0008-5472.CAN-04-3931]

9 **Szotek PP**, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, Dombkowski D, Preffer F, Maclaughlin DT, Donahoe PK. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. *Proc Natl Acad Sci USA* 2006; **103**: 11154-11159 [PMID: 16849428 DOI: 10.1073/pnas.0603672103]

10 **Zhang S**, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008; **68**: 4311-4320 [PMID: 18519691 DOI: 10.1158/0008-5472.CAN-08-0364]

11 **Kong X**, Wang X, Xu W, Behera S, Hellermann G, Kumar A, Lockey RF, Mohapatra S, Mohapatra SS. Natriuretic peptide receptor a as a novel anticancer target. *Cancer Res* 2008; **68**: 249-256 [PMID: 18172317 DOI: 10.1158/0008-5472.CAN-07-3086]

12 **Hashimoto H**, Sudo T, Mikami Y, Otani M, Takano M, Tsuda H, Itamochi H, Katabuchi H, Ito M, Nishimura R. Germ cell specific protein VASA is over-expressed in epithelial ovarian cancer and disrupts DNA damage-induced G2 checkpoint. *Gynecol Oncol* 2008; **111**: 312-319 [PMID: 18805576 DOI: 10.1016/j.ygyno.2008.08.014]

13 **Gao MQ**, Choi YP, Kang S, Youn JH, Cho NH. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. *Oncogene* 2010; **29**: 2672-2680 [PMID: 20190812 DOI: 10.1038/onc.2010.35]

14 **Kerr JB**, Duckett R, Myers M, Britt KL, Mladenovska T, Findlay JK. Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. *Reproduction* 2006; **132**: 95-109 [PMID: 16816336 DOI: 10.1530/rep.1.01128]

15 **Noce T**, Okamoto-Ito S, Tsunekawa N. Vasa homolog genes in mammalian germ cell development. *Cell Struct Funct* 2001; **26**: 131-136 [PMID: 11565805 DOI: 10.1247/csf.26.131]

16 **Gosden RG**. Germline stem cells in the postnatal ovary: is the ovary more like a testis? *Hum Reprod Update* 2004; **10**: 193-195 [PMID: 15140866 DOI: 10.1093/humupd/dmh023]

17 **Greenfeld C**, Flaws JA. Renewed debate over postnatal oogenesis in the mammalian ovary. *Bioessays* 2004; **26**: 829-832 [PMID: 15273985 DOI: 10.1002/bies.20094]

18 **Bristol-Gould SK**, Kreeger PK, Selkirk CG, Kilen SM, Mayo KE,

Shea LD, Woodruff TK. Fate of the initial follicle pool: empirical and mathematical evidence supporting its sufficiency for adult fertility. *Dev Biol* 2006; **298**: 149-154 [PMID: 16925987 DOI: 10.1016/j.ydbio.2006.06.023]

19 **Byskov AG**, Faddy MJ, Lemmen JG, Andersen CY. Eggs forever? *Differentiation* 2005; **73**: 438-446 [PMID: 16351687 DOI: 10.1111/j.1432-0436.2005.00045.x]

20 **Albertini DF**. Micromanagement of the ovarian follicle reserve--do stem cells play into the ledger? *Reproduction* 2004; **127**: 513-514 [PMID: 15129006 DOI: 10.1530/rep.1.00247]

21 **Motta PM**, Makabe S. Germ cells in the ovarian surface during fetal development in humans. A three-dimensional microanatomical study by scanning and transmission electron microscopy. *J Submicrosc Cytol* 1986; **18**: 271-290 [PMID: 3712511]

22 **Eppig JJ**, Wigglesworth K. Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components. *Biol Reprod* 2000; **63**: 1014-1023 [PMID: 10993822 DOI: 10.1095/biolreprod63.4.1014]

23 **Gosden RG**. Restitution of fertility in sterilized mice by transferring primordial ovarian follicles. *Hum Reprod* 1990; **5**: 499-504 [PMID: 2394782]

24 **Skaznik-Wikiel M**, Tilly JC, Lee HJ, Niikura Y, Kaneko-Tarui T, Johnson J, Tilly JL. Serious doubts over "Eggs forever?". *Differentiation* 2007; **75**: 93-99 [PMID: 17316379 DOI: 10.1111/j.1432-0436.2006.00117.x]

25 **Johnson J**, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* 2005; **122**: 303-315 [PMID: 16051153 DOI: 10.1016/j.cell.2005.06.031]

26 **Telfer EE**, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S, Tsafriri A. On regenerating the ovary and generating controversy. *Cell* 2005; **122**: 821-822 [PMID: 16179247 DOI: 10.1016/j.cell.2005.09.004]

27 **Eggan K**, Jurga S, Gosden R, Min IM, Wagers AJ. Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* 2006; **441**: 1109-1114 [PMID: 16799565 DOI: 10.1038/nature04929]

28 **Johnson J**, Skaznik-Wikiel M, Lee HJ, Niikura Y, Tilly JC, Tilly JL. Setting the record straight on data supporting postnatal oogenesis in female mammals. *Cell Cycle* 2005; **4**: 1471-1477 [PMID: 16222117 DOI: 10.4161/cc.4.11.2186]

29 **Zou K**, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol* 2009; **11**: 631-636 [PMID: 19363485 DOI: 10.1038/ncb1869]

30 **Pacchiarotti J**, Maki C, Ramos T, Marh J, Howerton K, Wong J, Pham J, Anorve S, Chow YC, Izadyar F. Differentiation potential of germ line stem cells derived from the postnatal mouse ovary. *Differentiation* 2010; **79**: 159-170 [PMID: 20138422 DOI: 10.1016/j.diff.2010.01.001]

31 **Oatley J**, Hunt PA. Of mice and (wo)men: purified oogonial stem cells from mouse and human ovaries. *Biol Reprod* 2012; **86**: 196 [PMID: 22402962 DOI: 10.1095/biolreprod.112.100297]

32 **Zhang H**, Zheng W, Shen Y, Adhikari D, Ueno H, Liu K. Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries. *Proc Natl Acad Sci USA* 2012; **109**: 12580-12585 [PMID: 22778414 DOI: 10.1073/pnas.1206600109]

33 **Lei L**, Spradling AC. Female mice lack adult germ-line stem cells but sustain oogenesis using stable primordial follicles. *Proc Natl Acad Sci USA* 2013; **110**: 8585-8590 [PMID: 23630252 DOI: 10.1073/pnas.1306189110]

34 **Woods DC**, White YA, Niikura Y, Kiatpongsan S, Lee HJ, Tilly JL. Embryonic stem cell-derived granulosa cells participate in ovarian follicle formation in vitro and in vivo. *Reprod Sci* 2013; **20**: 524-535 [PMID: 23536570 DOI: 10.1177/1933719112462632]

35 **Telfer EE**, Albertini DF. The quest for human ovarian stem cells. *Nat Med* 2012; **18**: 353-354 [PMID: 22395699 DOI: 10.1038/nm.2699]

36 **Xu Z**, Abbott A, Kopf GS, Schultz RM, Ducibella T. Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-trisphosphate sensitivity. *Biol Reprod* 1997; **57**: 743-750 [PMID: 9314575 DOI: 10.1095/biolreprod57.4.743]

37 **Zhao R**, Xuan Y, Li X, Xi R. Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. *Aging Cell* 2008; **7**: 344-354 [PMID: 18267001 DOI: 10.1111/j.1474-9726.2008.00379.x]

38 **Zhang X**, Ebata KT, Robaire B, Nagano MC. Aging of male germ line stem cells in mice. *Biol Reprod* 2006; **74**: 119-124 [PMID: 16177220 DOI: 10.1095/biolreprod.105.045591]

39 **Park ES**, Tilly JL. Use of DEAD-box polypeptide-4 (Ddx4) gene promoter-driven fluorescent reporter mice to identify mitotically active germ cells in post-natal mouse ovaries. *Mol Hum Reprod* 2015; **21**: 58-65 [PMID: 25147160]

40 **Al-Hajj M**, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene* 2004; **23**: 7274-7282 [PMID: 15378087 DOI: 10.1038/sj.onc.1207947]

41 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]

42 **Godwin AK**, Testa JR, Hamilton TC. The biology of ovarian cancer development. *Cancer* 1993; **71**: 530-536 [PMID: 8420673 DOI: 10.1002/cncr.2820710207]

43 **Yan HC**, Fang LS, Xu J, Qiu YY, Lin XM, Huang HX, Han QY. The identification of the biological characteristics of human ovarian cancer stem cells. *Eur Rev Med Pharmacol Sci* 2014; **18**: 3497-3503 [PMID: 25491627]

44 **Flesken-Nikitin A**, Hwang CI, Cheng CY, Michurina TV, Enikolopov G, Nikitin AY. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature* 2013; **495**: 241-245 [PMID: 23467088 DOI: 10.1038/nature11979]

45 **Ferrandina G**, Bonanno G, Pierelli L, Perillo A, Procoli A, Mariotti A, Corallo M, Martinelli E, Rutella S, Paglia A, Zannoni G, Mancuso S, Scambia G. Expression of CD133-1 and CD133-2 in ovarian cancer. *Int J Gynecol Cancer* 2008; **18**: 506-514 [PMID: 17868344 DOI: 10.1111/j.1525-1438.2007.01056.x]

46 **Curley MD**, Therrien VA, Cummings CL, Sergent PA, Koulouris CR, Friel AM, Roberts DJ, Seiden MV, Scadden DT, Rueda BR, Foster R. CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. *Stem Cells* 2009; **27**: 2875-2883 [PMID: 19816957 DOI: 10.1002/stem.236]

47 **Stewart JM**, Shaw PA, Gedye C, Bernardini MQ, Neel BG, Ailles LE. Phenotypic heterogeneity and instability of human ovarian tumor-initiating cells. *Proc Natl Acad Sci USA* 2011; **108**: 6468-6473 [PMID: 21451132 DOI: 10.1073/pnas.1005529108]

48 **Shi MF**, Jiao J, Lu WG, Ye F, Ma D, Dong QG, Xie X. Identification of cancer stem cell-like cells from human epithelial ovarian carcinoma cell line. *Cell Mol Life Sci* 2010; **67**: 3915-3925 [PMID: 20549538 DOI: 10.1007/s00018-010-0420-9]

49 **Bonnet D**, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737 [PMID: 9212098 DOI: 10.1038/nm0797-730]

50 **Blair A**, Hogge DE, Sutherland HJ. Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR-. *Blood* 1998; **92**: 4325-4335 [PMID: 9834239]

51 **Krivtsov AV**, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR, Armstrong SA. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* 2006; **442**: 818-822 [PMID: 16862118 DOI: 10.1038/nature04980]

52 **Baba T**, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T, Huang Z, Bentley RC, Mori S, Fujii S, Marks JR, Berchuck A, Murphy SK. Epigenetic regulation of CD133 and tumorigenicity

of CD133+ ovarian cancer cells. *Oncogene* 2009; **28**: 209-218 [PMID: 18836486 DOI: 10.1038/onc.2008.374]

53 **Ismail RS**, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC, Chang DD. Differential gene expression between normal and tumor-derived ovarian epithelial cells. *Cancer Res* 2000; **60**: 6744-6749 [PMID: 11118061]

54 **Kim KH**, Kang YJ, Jo JO, Ock MS, Moon SH, Suh DS, Yoon MS, Park ES, Jeong N, Eo WK, Kim HY, Cha HJ. DDX4 (DEAD box polypeptide 4) colocalizes with cancer stem cell marker CD133 in ovarian cancers. *Biochem Biophys Res Commun* 2014; **447**: 315-322 [PMID: 24727449 DOI: 10.1016/j.bbrc.2014.03.144]

55 **Fearon ER**. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011; **6**: 479-507 [PMID: 21090969 DOI: 10.1146/annurev-pathol-011110-130235]

56 **Sjöblom T**, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. *Science* 2006; **314**: 268-274 [PMID: 16959974 DOI: 10.1126/science.1133427]

57 **Rennie PS**, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. *Cancer Metastasis Rev* 1998-1999; **17**: 401-409 [PMID: 10453284 DOI: 10.1023/A: 1006121219097]

58 **Bakker J**, Lin X, Nelson WG. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. *J Biol Chem* 2002; **277**: 22573-22580 [PMID: 11960994 DOI: 10.1074/jbc.M203009200]

59 **Widschwendter M**, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW. Epigenetic stem cell signature in cancer. *Nat Genet* 2007; **39**: 157-158 [PMID: 17200673 DOI: 10.1038/ng1941]

60 **Fiegl H**, Windbichler G, Mueller-Holzner E, Goebel G, Lechner M, Jacobs IJ, Widschwendter M. HOXA11 DNA methylation--a novel prognostic biomarker in ovarian cancer. *Int J Cancer* 2008; **123**: 725-729 [PMID: 18478570 DOI: 10.1002/ijc.23563]

61 **Ibanez de Caceres I**, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, Bergman C, Ehya H, Eisenberg BL, Cairns P. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. *Cancer Res* 2004; **64**: 6476-6481 [PMID: 15374957]

62 **Aydoğdu E**, Katchy A, Tsouko E, Lin CY, Haldosén LA, Helguero L, Williams C. MicroRNA-regulated gene networks during mammary cell differentiation are associated with breast cancer. *Carcinogenesis* 2012; **33**: 1502-1511 [PMID: 22562546 DOI: 10.1093/carcin/bgs161]

63 **Liu C**, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D, Tang DG. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat Med* 2011; **17**: 211-215 [PMID: 21240262 DOI: 10.1038/nm.2284]

64 **Xie Z**, Cao L, Zhang J. miR-21 modulates paclitaxel sensitivity and hypoxia-inducible factor-1α expression in human ovarian cancer cells. *Oncol Lett* 2013; **6**: 795-800 [PMID: 24137413]

65 **Guo LM**, Pu Y, Han Z, Liu T, Li YX, Liu M, Li X, Tang H. MicroRNA-9 inhibits ovarian cancer cell growth through regulation of NF-kappaB1. *FEBS J* 2009; **276**: 5537-5546 [PMID: 19702828 DOI: 10.1111/j.1742-4658.2009.07237.x]

66 **Li Z**, Hu S, Wang J, Cai J, Xiao L, Yu L, Wang Z. MiR-27a modulates MDR1/P-glycoprotein expression by targeting HIPK2 in human ovarian cancer cells. *Gynecol Oncol* 2010; **119**: 125-130 [PMID: 20624637 DOI: 10.1016/j.ygyno.2010.06.004]

67 **Iorio MV**, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007; **67**: 8699-8707 [PMID: 17875710]

68 **Notarianni E**. Reinterpretation of evidence advanced for neo-oogenesis in mammals, in terms of a finite oocyte reserve. *J Ovarian Res* 2011; **4**: 1 [PMID: 21211009 DOI: 10.1186/1757-2215-4-1]

69 **Tedesco M**, Farini D, De Felici M. Impaired meiotic competence in putative primordial germ cells produced from mouse embryonic stem cells. *Int J Dev Biol* 2011; **55**: 215-222 [PMID: 21671224 DOI: 10.1387/ijdb.103108mt]

70 **Novak I**, Lightfoot DA, Wang H, Eriksson A, Mahdy E, Höög C. Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. *Stem Cells* 2006; **24**: 1931-1936 [PMID: 16644921]

71 **Dyce PW**, Liu J, Tayade C, Kidder GM, Betts DH, Li J. In vitro and in vivo germ line potential of stem cells derived from newborn mouse skin. *PLoS One* 2011; **6**: e20339 [PMID: 21629667 DOI: 10.1371/journal.pone.0020339]

72 **Park ES**, Woods DC, Tilly JL. Bone morphogenetic protein 4 promotes mammalian oogonial stem cell differentiation via Smad1/5/8 signaling. *Fertil Steril* 2013; **100**: 1468-1475 [PMID: 23993924 DOI: 10.1016/j.fertnstert.2013.07.1978]

73 **Wang N**, Tilly JL. Epigenetic status determines germ cell meiotic commitment in embryonic and postnatal mammalian gonads. *Cell Cycle* 2010; **9**: 339-349 [PMID: 20009537 DOI: 10.4161/cc.9.2.10447]

74 **Woods DC**, Tilly JL. The next (re)generation of ovarian biology and fertility in women: is current science tomorrow's practice? *Fertil Steril* 2012; **98**: 3-10 [PMID: 22682028 DOI: 10.1016/j.fertnstert.2012.05.005]

75 **Zhang Y**, Zhao FJ, Chen LL, Wang LQ, Nephew KP, Wu YL, Zhang S. MiR-373 targeting of the Rab22a oncogene suppresses tumor invasion and metastasis in ovarian cancer. *Oncotarget* 2014; **5**: 12291-12303 [PMID: 25460499]

76 **Zhao M**, Sun J, Zhao Z. Distinct and competitive regulatory patterns of tumor suppressor genes and oncogenes in ovarian cancer. *PLoS One* 2012; **7**: e44175 [PMID: 22952919 DOI: 10.1371/journal.pone.0044175]

77 **Feng Q**, Deftereos G, Hawes SE, Stern JE, Willner JB, Swisher EM, Xi L, Drescher C, Urban N, Kiviat N. DNA hypermethylation, Her-2/neu overexpression and p53 mutations in ovarian carcinoma. *Gynecol Oncol* 2008; **111**: 320-329 [PMID: 18757082 DOI: 10.1016/j.ygyno.2008.07.036]

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MINIREVIEWS

# Placental-derived stem cells: Culture, differentiation and challenges

Maira S Oliveira, João B Barreto-Filho

Maira S Oliveira, João B Barreto-Filho, College of Veterinary Medicine, Veterinary School, Universidade Federal de Minas Gerais, Belo Horizonte, MG 30161-970, Brazil
Maira S Oliveira, João B Barreto-Filho, Department of Veterinary Medicine, Universidade Federal de Lavras, Lavras, MG 37200-000, Brazil
Author contributions: Oliveira MS and Barreto-Filho JB both contributed to this paper.
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Correspondence to: Maira S Oliveira, VMD, MSc, PhD, College of Veterinary Medicine, Veterinary School, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, Caixa Postal 567, Belo Horizonte, MG 30161-970,
Brazil. maira_oliveira@yahoo.com
Telephone: +55-31-34092000
Fax: +55-31-34092230
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## Abstract

Stem cell therapy is a promising approach to clinical healing in several diseases. A great variety of tissues (bone marrow, adipose tissue, and placenta) are potentially sources of stem cells. Placenta-derived stem cells (p-SCs) are in between embryonic and mesenchymal stem cells, sharing characteristics with both, such as non-carcinogenic status and property to differentiate in all embryonic germ layers. Moreover, their use is not ethically restricted as fetal membranes are considered medical waste after birth. In this context, the present review will be focused on the biological properties, culture and potential cell therapy uses of placental-derived stem cells. Immunophenotype characterization, mainly for surface marker expression, and basic principles of p-SC isolation and culture (mechanical separation or enzymatic digestion of the tissues, the most used culture media, cell plating conditions) will be presented. In addition, some preclinical studies that were performed in different medical areas will be cited, focusing on neurological, liver, pancreatic, heart, muscle, pulmonary, and bone diseases and also in tissue engineering field. Finally, some challenges for stem cell therapy applications will be highlighted. The understanding of the mechanisms involved in the p-SCs differentiation and the achievement of pure cell populations (after differentiation) are key points that must be clarified before bringing the preclinical studies, performed at the bench, to the medical practice.

**Key words:** Fetal membrane; Placenta; Embryonic stem cells; Mesenchymal stem cells; Cell therapy

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**Core tip:** Fetal membranes are a source of stem cells, namely placenta-derived stem cells (p-SCs), which are in between embryonic and mesenchymal stem cells sharing characteristics with both, such as the capacity to differentiate in all germ layers and the dearth of tumorogenicity. Many preclinical studies have been investigated the potential of p-SC use in different medical areas, such as neurology, cardiology,

orthopedics, gastroenterology, and tissue engineering, showing promising results, but also some drawbacks. The present review will focus on different aspects of biological properties and potential clinical uses of p-SCs.

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## INTRODUCTION

Stem cell therapy is a promising approach to clinical healing in several diseases. Neurology[1], orthopedics[2] and cardiology[3] are just a few medical branches in which the benefits of stem cell therapy have been mentioned. The prolific capacity of stem cells (either embryonic or mesenchymal), taken together with the ability to generate different cell lineages and its self-renewal property are the major features of these cells that allow their use and manipulation in biology and medicine[4,5]. A great variety of tissues (bone marrow, adipose tissue, and endometrium) are potentially sources of stem cells and among them the placenta is a transient organ from which it is possible to obtain mesenchymal stem cells[5].

The term placenta constitutes a very reliable rich source of fetal mesenchymal stem cells[6-9]. These cells are capable of differentiating into multiple different cell types and have immunological properties that suggest their use in an allogenic transplantation setting[10-12].

In this respect, it is important to remember that placenta has a fundamental role in maintaining fetomaternal tolerance and, therefore, the immunomodulatory properties of these cells have been investigated with the aim of exploring their applicability in cell therapy-based treatments[11-13]. Their recovery does not involve any invasive procedures for the donor and their use does not create any ethical issue. In addition, the fact that placenta is generally discarded after birth as medical waste and is available in large supplies, makes placental-derived stem cells (p-SCs) excellent candidates for cell therapy. The current review is focused on the biological properties, culture and potential cell therapy applications of p-SCs.

## P-SC

### *Biological properties*

Animal development is initiated by fertilization of the egg with sperm, which is immediately followed by mitotic cell divisions, or cleavages, to generate blastomeres. For eutherians, such as the mouse and human, the first cell differentiation event is the establishment of two distinct cell lineages: the trophectoderm (TE) and the inner cell mass (ICM)[14]. TE engages in implantation by directly interacting with the mother's uterus, and gives rise to tissues in the placenta. It is only after implantation that the three germ layers form from the ICM, which ultimately generates all the tissues in the animal body[14]. Pluripotency is a characteristic of the ICM and is related to the expression of octamer binding transcription factor 4 (Oct4). Oct4 is essential to prevent ICM from diverting towards the TE lineage. Another important transcription factor, caudal type homeobox 2 (Cdx2), is specifically expressed in TE. It was demonstrated that Cdx2 is necessary to repress the expression of Oct4 in TE[15]. Moreover, Cdx2 null embryos exhibit high incidence of apoptosis[16].

Embryonic stem cells (ESCs), which are ICM-derived cell lines, despite of showing pluripotency and self-renewal capacity, have ethical concerns restraints[4], because of their tumorigenic potential. On the other hand, mesenchymal stem cells (MSCs) are not subject of these ethical restraints, but show multipotency, that means a lower capacity to multiply and originate different cell lineages[5]. Surrogate systems to study early placental development in the human species have been tried using human and mouse embryonic stem cells under bone morphogenetic protein (BMP) 2/4 stimulus to trophoblast differentiation and spontaneous formation of embryoid bodies[17,18]. MSCs are found in a lot of tissues, like the dentary tissue, adipocytes and bone marrow which culture provide inadequate cell numbers and have limitations like donor site morbidity and the need of aspiration to collect them, besides being a well-established culture. Attempts to overcome these problems were tried with alternative sources of MSCs and cells isolated from reproductive organs showed to be very useful. Thus, ovaries, uterus (endometrium) and specially the placenta[19-21], because of its discarded as medical waste after delivery, are potential sources of MSCs that can be used without ethical restrictions.

The p-SCs, which are TE-derived cell lines, gather some features from adult and embryo cells: possible differentiation to originate each of germinative cells and absence of carcinogenicity[22-27]. In the First International Workshop on p-SCs terminologies standardization have been made[28], and according to the cell origin we should have aminiotic epithelial or mesenchymal stromal cells, chorionic mesenchymal stromal or trophoblastic cells. In the present review it will be considered p-SCs in general, referring to any kind of them.

### *Cellular characterization*

Immunophenotype characterization is the most common procedure used to distinguish different cell clusters, by means of surface marker expression, and some essays employed are immunolabeling[29] and flow cytometry[30]. The p-SCs show profiles merged with those found in embryonic and mesenchymal stem cells[26]. None of these three cell lineages (embryonic,

mesenchymal, and p-SC as well) express the clusters of differentiation (CD) 11b, 34, and 45. Regarding CD expression, p-SCs are positive for 29, 73 and 166, and negative for 11b, 31, 34, 45. In addition, they are positive for human leucocyte antigen (HLA) A, B, C and negative for HLA-DR, DP, DQ[26,31,32].

The p-SCs fulfill the essential characteristics to categorize MSC[33] and are capable to originate ectodermal, endodermal, and mesodermal (adipocytes, osteocytes, and chondrocytes) lineages *in vitro*[25,31]. Moreover, p-SCs express surface cell markers only expressed by embryo-derived cells, such as Oct-4, SRY (sex determining region Y)-box 2 (Sox 2), Nanog, stage-specific embryonic antigen (SSEA)-1, SSEA-4, germ cell tumor marker (GCTM)-2, Tra-1-60, and Tra-1-80[22-24]. High levels of Oct-4, Sox 2 and Nanog were detected in p-SCs using q-PCR method[25]. Musashi-1, vimentin, polysialylated-neural cell adhesion molecule, and Nestin were observed in cells from amniotic membrane, unlike other MSCs, when evaluated by immunofluorescence technique[34].

### Cell culture

Many different protocols for isolation of fetal membranes-derived stem cells are found in literature. For human beings and large animals, a separation of amniotic epithelial cells[23,35], amniotic mesenchymal cells[7] and chorionic mesenchymal cells[8] are possible, meanwhile for small animals, such as rodents, such characterization is not possible[31].

Fetal membranes are submitted to enzymatic disaggregation following different protocols, which differ among themselves particularly regarding enzymatic digestion and duration[7,8,23,31,35]. In every protocol, the cells are incubated with trypsin-EDTA or other enzymes, at 37 ℃, and then, are harvested through physical methods. Then cells are plated onto noncoated tissue culture dishes or flasks. Serum, like fetal bovine serum (FBS) ranging from 10% to 20%, must be added to the media. The most commonly used media are roswell park memorial institute (RPMI)-1640, Dulbecco's modified eagle medium (DMEM), Minimum essential medium (EMEM), and DMEM/F12. Also, media should be enriched with nonessential amino acids, L-glutamine, β-mercaptoethanol, sodium pyruvate, and antibiotics. Cells are raised at controlled conditions of air atmosphere and temperature. For maintenance of the cell culture, p-SCs must have their medium (such as DMEM or RPMI) changed every other day. To passage the cells, it should be used trypsin, and the cells are generally plated into T75 tissue culture flasks. Such culturing conditions are very similar with MSCs. On the other hand, ESCs need to be plated into mouse embryonic fibroblasts - MEFs - feeder layer or Matrigel™ and the medium (such as DMEM-F12 or mTeSR™-1) must be changed every single day. To passage the cells, it should be used collagenase type IV or dispase, and the cells are generally plated into 6-well plates.

Finally, p-SCs may be cryopreserved using different media containing various concentrations of dimethyl sulfoxide, FBS and DMEM. Viability and cell numbers must be evaluated after thawing.

## CELL THERAPY USING P-SC

### Basic considerations

Three characteristics are important for the clinical application of p-SCs: the lack of ethical restrictions, pluripotency and their low immunogenicity[12]. An explanation for this is the low expression of MHC class II molecules, allowing these cells to be effectively employed in transplants[26].

Regarding to cellular therapy we have to consider the existence of cell banking due to the large amount of cells needed. Thus, those cells must have essential properties like to keep stable in subcultures, availability, great prolificity, freezing resistance, and high viability after thawing. The p-SCs fulfill most of these requirements. It has been mentioned that p-SCs even at subculture 30 showed high cellular division rates keeping a steady karyotype[25]. Different from p-SCs and ESCs, all other MSCs do not neither replicate nor exhibit high levels of cellular survival around subcultures 8 to 10.

Considering the freezing process, p-SCs require ordinary media (like MSCs, unlike ESCs), show intermediate resistance to low temperature (in between ESCs and MSCs) and show great viability after frozen-thawing (like ESCs, unlike MSCs).

Some of the p-SC uses in research are listed in Table 1, at the end of this section.

### Neurological diseases

Human p-SC demonstrated neuroprotective effects after stroke in rats. Treatment of stroke with p-SCs (*via* intravenous administration) significantly increased vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and brain derived neurotrophic factor (BDNF) levels in the ischemic brain compared to controls (dextran vehicle or phosphate buffer saline) after middle cerebral artery occlusion[36].

In Alzheimer's disease, β-amyloid peptide is considered to be its root cause. Also, the neuroinflammatory process mediated by β-amyloid plaque-induced microglial cells and astrocytes contributes to Alzheimer's disease pathogenesis. Thus, it was demonstrated that p-SCs transplanted into an Alzheimer's disease mouse model modulated the properties of microglial cells toward a β-amyloid peptide plaque-reducing anti-inflammatory response. Moreover, p-SCs injected mice, compared to phosphate-buffered saline controls, had higher levels of β-amyloid degrading enzymes, reduced levels of proinflammatory cytokines, increased levels of anti-inflammatory cytokines (TGF-β and IL-10), slower progression of Alzheimer's pathology, and

**Table 1 Preclinical studies using placental-derived stem cells for different disease models**

| Disease | Animal model | Cells | Ref. |
|---|---|---|---|
| Stroke | Rats | p-SC | [36] |
| Alzheimer | Mouse | p-SC | [37] |
| Injured liver model | Rat | p-SC | [39] |
| Myocardial infarction | Rat | amniotic membrane fragment | [43] |
| Myocardial infarction | Pig | p-SC | [44] |
| Lung fibrosis model | Mouse | p-SC | [47] |
| Idiopathic pulmonary fibrosis | Human (trial study) | p-SC | [48] |
| Osteogenesis imperfecta | Murine | p-SC | [49] |
| Bone lytic lesions | Mice | p-SC | [50] |
| Bone disease | rabbit | p-SC + scaffold | [51] |

p-SC: Placenta-derived stem cell.

improved memory function[37].

### Liver disease

Cell therapy for liver diseases has also been investigated. Among other sources of MSCs, p-SCs showed the greatest potential for hepatogenic differentiation and proliferation *in vitro*. The p-SCs, mainly those from chorionic plate, expressed higher levels of hepatocyte growth factor after differentiation[38].

In another study, chorionic-plate derived mesenchymal stem cells isolated from placenta could trigger autophagy to enhance regeneration in carbon tetrachloride injured rat liver model. After p-SC transplantation they observed reduction in apoptosis (caspase activity) and increasing levels for autophagy, survival and regeneration in liver cells[39].

### Pancreatic disease

Human placenta-derived mesenchymal stem cells (p-SCs) have the potential to differentiate into insulin producing cells[40,41]. Also, p-SCs can form islet-like cell clusters which are capable of restoring normoglycemia when transplanted into streptozotocin-induced diabetic Balb/C mice[42].

### Heart disease

In order to evaluate whether human amniotic membrane could limit postischemic cardiac injury, a fragment was applied onto the left ventricle of rats that had undergone ischemia through left anterior descending coronary artery ligation. The authors observed that the amniotic membrane fragment onto ischemic rat hearts could significantly reduce postischemic cardiac dysfunction once the rats showed higher values of left ventricle ejection fraction, fractional shortening and wall thickening on echocardiographic examinations[43].

In another study of myocardial infarction, pretreated (with a hyaluronan mixed ester of butyric and retinoic acid - HBR) p-SCs were intramyocardial injected in a pig model. Treated animals showed smaller infarct scar size and a significant improvement of the end-systolic wall thickening and circumferential shortening of the infarct border zone evaluated by magnetic resonance imaging[44].

### Muscle disease

First-trimester chorionic-villi-derived cells were evaluated for potential therapeutic use in Duchenne muscular dystrophy, which is a X-linked disorder characterized by the absence of dystrophin at the sarcolemma of muscle fibers. The p-SCs efficiently differentiated into myotubes after myogenic induction, at which point Nanog and Sox2 were down-regulated, whereas MyoD, myogenin, desmin and dystrophin were up-regulated. The p-SCs could be efficiently directed to differentiate *in vitro* into skeletal muscle cells that express dystrophin as the last stage marker of myogenic differentiation[45].

The myogenic differentiation of fetal stem cells (p-SC, amniotic fluid stem cells, cord blood and wharton's jelly cells), were already reviewed and considerations about limitations and perspectives for therapeutically use were done[46].

### Pulmonary disease

On a mouse model of bleomycin-induced lung fibrosis, cellular therapy using p-SCs promoted a decrease in neutrophil infiltration and a significant reduction in the severity of fibrosis, compared to control[47].

On a single centre, non-randomized, dose escalation phase 1b trial study, human patients with moderately to severe idiopathic pulmonary fibrosis received p-SCs *via* peripheral vein and they were evaluated follow up 6 mo. The variables analyzed were lung function (forced vital capacity and diffusing capacity for carbon monoxide), 6-min walk distance, gas exchange (assessed by resting PaO2), and lung fibrosis score (assessed by high-resolution computed tomography chest). Compared to baseline, at 6 mo all parameters were unchanged, with no evidence of worsening fibrosis and no side effects reported in the patients who had received p-SC injections[48].

### Bone disease

Human fetal early chorionic stem cells (p-SCs) treatment was studied in a murine osteogenesis imperfecta model. It was demonstrated that intraperitoneal injection of p-SCs in osteogenesis imperfect neonates reduced fractures, increased bone ductility and bone volume, increased the numbers of hypertrophic chondrocytes, and upregulated endogenous genes involved in endochondral and intramembranous ossification[49].

Regenerative medicine using p-SC for bone disease (large lytic lesions) secondary to multiple myeloma was investigated. The authors founded p-SCs inhibitory effects on myeloma bone disease and tumor growth were dose-dependent and intrabone engraftment of p-SCs inhibited bone disease and tumor growth in mice severe combined immunodeficiency-rab model.

The p-SCs also promoted apoptosis in osteoclast precursors and inhibited their differentiation, indicating a promising therapeutic approach for myeloma osteolysis using cytotherapy[50].

### *Tissue engenieering*

The utility of p-SCs for bone tissue engineering has been investigated using different biomaterials and showing promising results. Transplantation of human p-SCs grown in a silk fibroin/hydroxyapatite scaffold into injured radius segmental bone in rabbits enhance tissue repair[51]. A combination of p-SCs and polylactide/poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) fibrous scaffolds promoted good cellular response and excellent osteogenic potential *in vitro*[52].

An elastic scaffold, obtained combining a poly-(ether)urethane-polydimethylsiloxane (PEtU-PDMS) semi-interpenetrating polymeric network (s-IPN) with fibrin, was used as a substrate for *in vitro* studies of human p-SC growth and differentiation. The s-IPN PEtU-PDMS/fibrin combined scaffold allowed a better proliferation and metabolic activity of p-SC cultured up to 14 d, compared to the ones grown on plastic dishesand also sustained the beginning of p-SCs differentiation process towards a cardiomyogenic lineage[53].

As an alternative to corneal transplantation, researchers evaluated a biomaterial derived from fetal membranes to promote corneal repair. The pericellular matrix of decidua-derived mesenchymal cells was demonstrated to provide a xeno-free substrate for efficient culture of human corneal endothelial cells. The matrix enhanced corneal cell attachment, promoted cell proliferation, and suppressed apoptosis, offering a viable *in vitro* expansion protocol for human corneal endothelial cells[54].

Cell-based therapy, using p-SC, combined with bioactive materials improved bone regeneration prior to dental implant. Amniotic epithelial cells, loaded on a calcium-phosphate synthetic bone substitute, displayed a reduced fibrotic reaction, a limited inflammatory response and an accelerated process of angiogenesis. In addition, the presence of p-SCs significantly stimulated osteogenesis by enhancing bone deposition, as suggested by the presence of p-SCs entrapped within the newly deposited osteoid matrix and by their ability to switch-on the expression of osteocalcin when transplanted into host tissues[2].

## CHALLENGES

A routine medical practice using stem cells is an exciting promise. And for this reason, any preclinical study regarding cell therapy must be considered cautiously. Besides the rapid evolution in differentiating stem cells into many different cell types, all protocols result in a mix cell preparations and how to get specialized cells in sufficient amount and purity is still a challenge. So, the really understanding of all the mechanisms from culturing stem cells to transplanting differentiated ones into a patient is of great importance in order to let the dream comes true.

## CONCLUSION

In summary, either the fetal or the maternal components of the placenta may be considered potential sources of stem cells once they share features from both cells of embryonic and mesenchymal origin. Phenotypically, these cells show common characteristics of embryo-derived and adult stem cells and do not express hematopoietic cell markers CD11b, CD 34 and CD 45. Different media to culture and stimuli to onward differentiation in p-SCs are under investigation and they have been used on research trials of cell therapy with promising results. Furthermore, it is important to get enough purity cells and to understand the mechanism of differentiation and engraftment of p-SC to improve cell-based therapy.

## REFERENCES


1 **Titomanlio L**, Kavelaars A, Dalous J, Mani S, El Ghouzzi V, Heijnen C, Baud O, Gressens P. Stem cell therapy for neonatal brain injury: perspectives and challenges. *Ann Neurol* 2011; **70**: 698-712 [PMID: 22162055 DOI: 10.1002/ana.22518]

2 **Barboni B**, Mangano C, Valbonetti L, Marruchella G, Berardinelli P, Martelli A, Muttini A, Mauro A, Bedini R, Turriani M, Pecci R, Nardinocchi D, Zizzari VL, Tetè S, Piattelli A, Mattioli M. Synthetic bone substitute engineered with amniotic epithelial cells enhances bone regeneration after maxillary sinus augmentation. *PLoS One* 2013; **8**: e63256 [PMID: 23696804 DOI: 10.1371/journal.pone.0063256]

3 **Ventura C**, Cantoni S, Bianchi F, Lionetti V, Cavallini C, Scarlata I, Foroni L, Maioli M, Bonsi L, Alviano F, Fossati V, Bagnara GP, Pasquinelli G, Recchia FA, Perbellini A. Hyaluronan mixed esters of butyric and retinoic Acid drive cardiac and endothelial fate in term placenta human mesenchymal stem cells and enhance cardiac repair in infarcted rat hearts. *J Biol Chem* 2007; **282**: 14243-14252 [PMID: 17363374 DOI: 10.1074/jbc.M609350200]

4 **Solter D**. From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. *Nat Rev Genet* 2006; **7**: 319-327 [PMID: 16534514 DOI: 10.1038/nrg1827]

5 **Chamberlain G**, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007; **25**: 2739-2749 [PMID: 17656645 DOI: 10.1634/stemcells.2007-0197]

6 **Wulf GG**, Viereck V, Hemmerlein B, Haase D, Vehmeyer K, Pukrop T, Glass B, Emons G, Trümper L. Mesengenic progenitor cells derived from human placenta. *Tissue Eng* 2004; **10**: 1136-1147 [PMID: 15363170]

7 **Wolbank S**, van Griensven M, Grillari-Voglauer R, Peterbauer-Scherb A. Alternative sources of adult stem cells: human amniotic membrane. *Adv Biochem Eng Biotechnol* 2010; **123**: 1-27 [PMID: 20237903]

8 **Rus Ciucă D**, Soriţău O, Suşman S, Pop VI, Mihu CM. Isolation and characterization of chorionic mesenchyal stem cells from the placenta. *Rom J Morphol Embryol* 2011; **52**: 803-808 [PMID: 21892522]

9 **Karlsson H**, Erkers T, Nava S, Ruhm S, Westgren M, Ringdén O. Stromal cells from term fetal membrane are highly suppressive in allogeneic settings in vitro. *Clin Exp Immunol* 2012; **167**: 543-555 [PMID: 22288598 DOI: 10.1111/j.1365-2249.2011.04540.x]

10 **Miao Z**, Jin J, Chen L, Zhu J, Huang W, Zhao J, Qian H, Zhang X. Isolation of mesenchymal stem cells from human placenta:

comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int* 2006; **30**: 681-687 [PMID: 16870478]

11 **Apps R**, Murphy SP, Fernando R, Gardner L, Ahad T, Moffett A. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology* 2009; **127**: 26-39 [PMID: 19368562 DOI: 10.1111/j.1365-2567.2008.03019.x]

12 **Parolini O**, Caruso M. Review: Preclinical studies on placenta-derived cells and amniotic membrane: an update. *Placenta* 2011; **32** Suppl 2: S186-S195 [PMID: 21251712 DOI: 10.1016/j.placenta.2010.12.016]

13 **Bailo M**, Soncini M, Vertua E, Signoroni PB, Sanzone S, Lombardi G, Arienti D, Calamani F, Zatti D, Paul P, Albertini A, Zorzi F, Cavagnini A, Candotti F, Wengler GS, Parolini O. Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation* 2004; **78**: 1439-1448 [PMID: 15599307 DOI: 10.1097/01.TP.0000144606.84234.49]

14 **Marikawa Y**, Alarcón VB. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. *Mol Reprod Dev* 2009; **76**: 1019-1032 [PMID: 19479991 DOI: 10.1002/mrd.21057]

15 **Strumpf D**, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 2005; **132**: 2093-2102 [PMID: 15788452]

16 **Rossant J**. Developmental biology: A mouse is not a cow. *Nature* 2011; **471**: 457-458 [PMID: 21430771 DOI: 10.1038/471457]

17 **Golos TG**, Giakoumopoulos M, Gerami-Naini B. Review: Trophoblast differentiation from human embryonic stem cells. *Placenta* 2013; **34** Suppl: S56-S61 [PMID: 23261342 DOI: 10.1016/j.placenta.2012.11.019]

18 **Hayashi Y**, Furue MK, Tanaka S, Hirose M, Wakisaka N, Danno H, Ohnuma K, Oeda S, Aihara Y, Shiota K, Ogura A, Ishiura S, Asashima M. BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin. *In Vitro Cell Dev Biol Anim* 2010; **46**: 416-430 [PMID: 20033790 DOI: 10.1007/s11626-009-9266-6]

19 **Mohr S**, Portmann-Lanz CB, Schoeberlein A, Sager R, Surbek DV. Generation of an osteogenic graft from human placenta and placenta-derived mesenchymal stem cells. *Reprod Sci* 2010; **17**: 1006-1015 [PMID: 20940246 DOI: 10.1177/1933719110377471]

20 **Shin KS**, Lee HJ, Jung J, Cha DH, Kim GJ. Culture and in vitro hepatogenic differentiation of placenta-derived stem cells, using placental extract as an alternative to serum. *Cell Prolif* 2010; **43**: 435-444 [PMID: 20887550 DOI: 10.1111/j.1365-2184.2010.00693.x]

21 **Vidane AS**, Zomer HD, Oliveira BM, Guimarães CF, Fernandes CB, Perecin F, Silva LA, Miglino MA, Meirelles FV, Ambrósio CE. Reproductive stem cell differentiation: extracellular matrix, tissue microenvironment, and growth factors direct the mesenchymal stem cell lineage commitment. *Reprod Sci* 2013; **20**: 1137-1143 [PMID: 23420825 DOI: 10.1177/1933719113477484]

22 **Fujimoto KL**, Miki T, Liu LJ, Hashizume R, Strom SC, Wagner WR, Keller BB, Tobita K. Naive rat amnion-derived cell transplantation improved left ventricular function and reduced myocardial scar of postinfarcted heart. *Cell Transplant* 2009; **18**: 477-486 [PMID: 19622235 DOI: 10.3727/096368909788809785]

23 **Manuelpillai U**, Tchongue J, Lourensz D, Vaghjiani V, Samuel CS, Liu A, Williams ED, Sievert W. Transplantation of human amnion epithelial cells reduces hepatic fibrosis in immunocompetent $CCl_4$-treated mice. *Cell Transplant* 2010; **19**: 1157-1168 [PMID: 20447339 DOI: 10.3727/096368910X504496]

24 **Moodley Y**, Ilancheran S, Samuel C, Vaghjiani V, Atienza D, Williams ED, Jenkin G, Wallace E, Trounson A, Manuelpillai U. Human amnion epithelial cell transplantation abrogates lung fibrosis and augments repair. *Am J Respir Crit Care Med* 2010; **182**: 643-651 [PMID: 20522792 DOI: 10.1164/rccm.201001-0014OC]

25 **Sabapathy V**, Ravi S, Srivastava V, Srivastava A, Kumar S. Long-term cultured human term placenta-derived mesenchymal stem cells of maternal origin displays plasticity. *Stem Cells Int* 2012; **2012**: 174328 [PMID: 22550499 DOI: 10.1155/2012/174328]

26 **Li X**, Bai J, Ji X, Li R, Xuan Y, Wang Y. Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation. *Int J Mol Med* 2014; **34**: 695-704 [PMID: 24970492 DOI: 10.3892/ijmm.2014.1821]

27 **Vidane AS**, Souza AF, Sampaio RV, Bressan FF, Pieri NC, Martins DS, Meirelles FV, Miglino MA, Ambrósio CE. Cat amniotic membrane multipotent cells are nontumorigenic and are safe for use in cell transplantation. *Stem Cells Cloning* 2014; **7**: 71-78 [PMID: 25249758 DOI: 10.2147/SCCAA.S67790]

28 **Parolini O**, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008; **26**: 300-311 [PMID: 17975221 DOI: 10.1634/stemcells.2007-0594]

29 **Oliveira MS**, Carvalho JL, Campos AC, Gomes DA, de Goes AM, Melo MM. Doxorubicin has in vivo toxicological effects on ex vivo cultured mesenchymal stem cells. *Toxicol Lett* 2014; **224**: 380-386 [PMID: 24291741 DOI: 10.1016/j.toxlet.2013.11.023]

30 **Carvalho JL**, Braga VB, Melo MB, Campos AC, Oliveira MS, Gomes DA, Ferreira AJ, Santos RA, Goes AM. Priming mesenchymal stem cells boosts stem cell therapy to treat myocardial infarction. *J Cell Mol Med* 2013; **17**: 617-625 [PMID: 23490190 DOI: 10.1111/jcmm.12036]

31 **Ishikane S**, Ohnishi S, Yamahara K, Sada M, Harada K, Mishima K, Iwasaki K, Fujiwara M, Kitamura S, Nagaya N, Ikeda T. Allogeneic injection of fetal membrane-derived mesenchymal stem cells induces therapeutic angiogenesis in a rat model of hind limb ischemia. *Stem Cells* 2008; **26**: 2625-2633 [PMID: 18669910 DOI: 10.1634/stemcells.2008-0236]

32 **Kranz A**, Wagner DC, Kamprad M, Scholz M, Schmidt UR, Nitzsche F, Aberman Z, Emmrich F, Riegelsberger UM, Boltze J. Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats. *Brain Res* 2010; **1315**: 128-136 [PMID: 20004649 DOI: 10.1016/j.brainres.2009.12.001]

33 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

34 **Kong XY**, Cai Z, Pan L, Zhang L, Shu J, Dong YL, Yang N, Li Q, Huang XJ, Zuo PP. Transplantation of human amniotic cells exerts neuroprotection in MPTP-induced Parkinson disease mice. *Brain Res* 2008; **1205**: 108-115 [PMID: 18353283 DOI: 10.1016/j.brainres.2008.02.040]

35 **Sakuragawa N**, Thangavel R, Mizuguchi M, Hirasawa M, Kamo I. Expression of markers for both neuronal and glial cells in human amniotic epithelial cells. *Neurosci Lett* 1996; **209**: 9-12 [PMID: 8734897]

36 **Chen J**, Shehadah A, Pal A, Zacharek A, Cui X, Cui Y, Roberts C, Lu M, Zeitlin A, Hariri R, Chopp M. Neuroprotective effect of human placenta-derived cell treatment of stroke in rats. *Cell Transplant* 2013; **22**: 871-879 [PMID: 22469567]

37 **Kim KS**, Kim HS, Park JM, Kim HW, Park MK, Lee HS, Lim DS, Lee TH, Chopp M, Moon J. Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging* 2013; **34**: 2408-2420 [PMID: 23623603 DOI: 10.1016/j.neurobiolaging.2013.03.029]

38 **Lee HJ**, Jung J, Cho KJ, Lee CK, Hwang SG, Kim GJ. Comparison of in vitro hepatogenic differentiation potential between various placenta-derived stem cells and other adult stem cells as an alternative source of functional hepatocytes. *Differentiation* 2012; **84**: 223-231 [PMID: 22885322 DOI: 10.1016/j.diff.2012.05.007]

39 **Jung J**, Choi JH, Lee Y, Park JW, Oh IH, Hwang SG, Kim KS, Kim GJ. Human placenta-derived mesenchymal stem cells promote

hepatic regeneration in CCl4 -injured rat liver model via increased autophagic mechanism. *Stem Cells* 2013; **31**: 1584-1596 [PMID: 23592412 DOI: 10.1002/stem.1396]

40 **Chang CM**, Kao CL, Chang YL, Yang MJ, Chen YC, Sung BL, Tsai TH, Chao KC, Chiou SH, Ku HH. Placenta-derived multipotent stem cells induced to differentiate into insulin-positive cells. *Biochem Biophys Res Commun* 2007; **357**: 414-420 [PMID: 17433254 DOI: 10.1016/j.bbrc.2007.03.157]

41 **Sun NZ**, Ji HS. In vitro differentiation of human placenta-derived adherent cells into insulin-producing cells. *J Int Med Res* 2010; **37**: 400-406 [PMID: 19383234 DOI: 10.1177/147323000903700215]

42 **Kadam S**, Muthyala S, Nair P, Bhonde R. Human placenta-derived mesenchymal stem cells and islet-like cell clusters generated from these cells as a novel source for stem cell therapy in diabetes. *Rev Diabet Stud* 2010; **7**: 168-182 [PMID: 21060975 DOI: 10.1900/RDS.2010.7.168]

43 **Cargnoni A**, Di Marcello M, Campagnol M, Nassuato C, Albertini A, Parolini O. Amniotic membrane patching promotes ischemic rat heart repair. *Cell Transplant* 2009; **18**: 1147-1159 [PMID: 19650976 DOI: 10.3727/096368909X12483162196764]

44 **Simioniuc A**, Campan M, Lionetti V, Marinelli M, Aquaro GD, Cavallini C, Valente S, Di Silvestre D, Cantoni S, Bernini F, Simi C, Pardini S, Mauri P, Neglia D, Ventura C, Pasquinelli G, Recchia FA. Placental stem cells pre-treated with a hyaluronan mixed ester of butyric and retinoic acid to cure infarcted pig hearts: a multimodal study. *Cardiovasc Res* 2011; **90**: 546-556 [PMID: 21257613 DOI: 10.1093/cvr/cvr018]

45 **Arakawa R**, Aoki R, Arakawa M, Saito K. Human first-trimester chorionic villi have a myogenic potential. *Cell Tissue Res* 2012; **348**: 189-197 [PMID: 22370594 DOI: 10.1007/s00441-012-1340-9]

46 **Pozzobon M**, Franzin C, Piccoli M, De Coppi P. Fetal stem cells and skeletal muscle regeneration: a therapeutic approach. *Front Aging Neurosci* 2014; **6**: 222 [PMID: 25221507 DOI: 10.3389/fnagi.2014.00222]

47 **Cargnoni A**, Gibelli L, Tosini A, Signoroni PB, Nassuato C, Arienti D, Lombardi G, Albertini A, Wengler GS, Parolini O. Transplantation of allogeneic and xenogeneic placenta-derived cells reduces bleomycin-induced lung fibrosis. *Cell Transplant* 2009; **18**: 405-422 [PMID: 19622228 DOI: 10.3727/096368909788809857]

48 **Chambers DC**, Enever D, Ilic N, Sparks L, Whitelaw K, Ayres J, Yerkovich ST, Khalil D, Atkinson KM, Hopkins PM. A phase 1b study of placenta-derived mesenchymal stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2014; **19**: 1013-1018 [PMID: 25039426 DOI: 10.1111/resp.12343]

49 **Jones GN**, Moschidou D, Abdulrazzak H, Kalirai BS, Vanleene M, Osatis S, Shefelbine SJ, Horwood NJ, Marenzana M, De Coppi P, Bassett JH, Williams GR, Fisk NM, Guillot PV. Potential of human fetal chorionic stem cells for the treatment of osteogenesis imperfecta. *Stem Cells Dev* 2014; **23**: 262-276 [PMID: 24028330 DOI: 10.1089/scd.2013.0132]

50 **Li X**, Ling W, Pennisi A, Wang Y, Khan S, Heidaran M, Pal A, Zhang X, He S, Zeitlin A, Abbot S, Faleck H, Hariri R, Shaughnessy JD, van Rhee F, Nair B, Barlogie B, Epstein J, Yaccoby S. Human placenta-derived adherent cells prevent bone loss, stimulate bone formation, and suppress growth of multiple myeloma in bone. *Stem Cells* 2011; **29**: 263-273 [PMID: 21732484 DOI: 10.1002/2010]

51 **Jin J**, Wang J, Huang J, Huang F, Fu J, Yang X, Miao Z. Transplantation of human placenta-derived mesenchymal stem cells in a silk fibroin/hydroxyapatite scaffold improves bone repair in rabbits. *J Biosci Bioeng* 2014; **118**: 593-598 [PMID: 24894683 DOI: 10.1016/j.jbiosc.2014.05.001]

52 **Wang Y**, Guo G, Chen H, Gao X, Fan R, Zhang D, Zhou L. Preparation and characterization of polylactide/poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) hybrid fibers for potential application in bone tissue engineering. *Int J Nanomedicine* 2014; **9**: 1991-2003 [PMID: 24790439 DOI: 10.2147/IJN.S55318]

53 **Lisi A**, Briganti E, Ledda M, Losi P, Grimaldi S, Marchese R, Soldani G. A combined synthetic-fibrin scaffold supports growth and cardiomyogenic commitment of human placental derived stem cells. *PLoS One* 2012; **7**: e34284 [PMID: 22509287 DOI: 10.1371/journal.pone.0034284]

54 **Numata R**, Okumura N, Nakahara M, Ueno M, Kinoshita S, Kanematsu D, Kanemura Y, Sasai Y, Koizumi N. Cultivation of corneal endothelial cells on a pericellular matrix prepared from human decidua-derived mesenchymal cells. *PLoS One* 2014; **9**: e88169 [PMID: 24505413 DOI: 10.1371/journal.pone.0088169]

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ORIGINAL ARTICLE

Basic Study

# Fetal kidney stem cells ameliorate cisplatin induced acute renal failure and promote renal angiogenesis

Ashwani Kumar Gupta, Sachin H Jadhav, Naresh Kumar Tripathy, Soniya Nityanand

Ashwani Kumar Gupta, Sachin H Jadhav, Naresh Kumar Tripathy, Soniya Nityanand, Stem Cell Research Facility, Department of Hematology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow 226014, India

Author contributions: Gupta AK designed and performed the experiments and analyzed the data; Jadhav SH, Tripathy NK and Nityanand S designed the study and analyzed the data; Nityanand S provided the reagents and analytical tools; Gupta AK, Jadhav SH, Tripathy NK and Nityanand S wrote the manuscript.

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Data sharing: Technical appendix, statistical code, and dataset available with the corresponding author at soniya_nityanand@yahoo.co.in, who will provide a permanent, citable and open-access home for the dataset. No additional data are available (animal study).

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Correspondence to: Dr. Soniya Nityanand, MD, PhD, FNASc, FASc, Professor and Head, Department of Hematology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Rae Bareli Road, Lucknow 226014, India. soniya_nityanand@yahoo.co.in
Telephone: +91-522-2494291
Fax: +91-522-2668017

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## Abstract

**AIM:** To investigate whether fetal kidney stem cells (fKSC) ameliorate cisplatin induced acute renal failure (ARF) in rats and promote renal angiogenesis.

**METHODS:** The fKSC were isolated from rat fetuses of gestation day 16 and expanded *in vitro* up to 3rd passage. They were characterized for the expression of mesenchymal and renal progenitor markers by flow cytometry and immunocytochemistry, respectively. The *in vitro* differentiation of fKSC towards epithelial lineage was evaluated by the treatment with specific induction medium and their angiogenic potential by matrigel induced tube formation assay. To study the effect of fKSC in ARF, fKSC labeled with PKH26 were infused in rats with cisplatin induced ARF and, the blood and renal tissues of the rats were collected at different time points. Blood biochemical parameters were studied to evaluate renal function. Renal tissues were evaluated for renal architecture, renal cell proliferation and angiogenesis by immunohistochemistry, renal cell apoptosis by terminal deoxynucleotidyl transferase nick-end labeling assay and early expression of angiogenic molecules *viz*. vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1$\alpha$ and endothelial nitric oxide synthase (eNOS) by western blot.

**RESULTS:** The fKSC expressed mesenchymal markers *viz*. CD29, CD44, CD73, CD90 and CD105 as well as

renal progenitor markers *viz*. Wt1, Pax2 and Six2. They exhibited a potential to form CD31 and Von Willebrand factor expressing capillary-like structures and could be differentiated into cytokeratin (CK)18 and CK19 positive epithelial cells. Administration of fKSC in rats with ARF as compared to administration of saline alone, resulted in a significant improvement in renal function and histology on day 3 (2.33 ± 0.33 *vs* 3.50 ± 0.34, $P < 0.05$) and on day 7 (0.83 ± 0.16 *vs* 2.00 ± 0.25, $P < 0.05$). The infused PKH26 labeled fKSC were observed to engraft in damaged renal tubules and showed increased proliferation and reduced apoptosis ($P < 0.05$) of renal cells. The kidneys of fKSC as compared to saline treated rats had a higher capillary density on day 3 [13.30 ± 1.54 *vs* 7.10 ± 1.29, capillaries/high-power fields (HPF), $P < 0.05$], and on day 7 (21.10 ± 1.46 *vs* 15.00 ± 1.30, capillaries/HPF, $P < 0.05$). In addition, kidneys of fKSC treated rats had an up-regulation of angiogenic proteins hypoxia-inducible factor-1$\alpha$, VEGF and eNOS on day 3 ($P < 0.05$).

**CONCLUSION:** Our study shows that fKSC ameliorate cisplatin induced ARF in rats and promote renal angiogenesis, which may be an important therapeutic mechanism of these stem cells in the disease.

**Key words:** Fetal kidney stem cells; Mesenchymal and renal progenitor markers; Acute renal failure; Stem cell therapy; Angiogenesis

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**Core tip:** This study provides novel data on the therapeutic effect of culture expanded fetal kidney stem cells (fKSC) in acute renal failure (ARF). The fKSC represent primitive renal stem cells that express mesenchymal and renal progenitor markers. They exhibit *in vitro* angiogenesis and potential to differentiate into renal epithelial cells. On administration in ARF rats, they rapidly improve renal function and histology. The therapeutic effects of fKSC are accompanied with increased capillary density in kidney tissues suggesting that induction of renal angiogenesis may be an important therapeutic mechanism of these stem cells.

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## INTRODUCTION

Acute renal failure (ARF) is characterized by rapid loss of renal function due to damage of renal tubular epithelial cells by ischemia, nephrotoxins or other means. Some cases of ARF spontaneously recover from renal injury while in others the recovery process is either delayed or does not occur at all, leading to chronic kidney disease (CKD), which is associated with high morbidity and mortality[1,2]. Thus, it is very important to restore normal structure and function of the kidney after ARF using a suitable regenerative therapy in order to prevent its progression into CKD[3]. Recent data suggest that injury of endothelial cells of small peritubular arterioles and capillaries plays a crucial role in the pathogenesis of ARF by mediating hypoxia and impairment of renal perfusion that eventually results in injury of renal tubular epithelial cells and thereby loss of renal function[4-6]. Furthermore, reduction in renal capillary density following ARF may perpetuate the tubular epithelial injury leading to CKD[7,8]. These observations suggest that neovascularization of the kidney and regeneration of damaged renal tubular epithelial cells may be potentially effective in recovery from ARF.

During the past decade, stem cell based regenerative therapy has emerged as a promising therapeutic strategy for ARF and stem cells derived from various sources are being extensively explored for the treatment of ARF[9,10]. However, a suitable stem cell type for therapeutic application in clinical ARF has not yet been identified. Fetal kidney stem cells (fKSC) represent a novel stem cell type for treatment of ARF because they are multipotent stem cells with an inherent ability to differentiate into cells of renal lineage. In addition, few studies have shown that embryonic or fetal kidney also contains a subpopulation of endothelial and epithelial progenitors[11-13] and hence fKSC may have an important role in neovascularization and the regeneration of the damaged kidney. However, there is a paucity of data on therapeutic effect of fKSC in ARF and no information is available about angiogenic role of these stem cells in mediating their therapeutic effect in the disease.

Therefore, the aim of the present study was to investigate the therapeutic effect of fKSC in cisplatin induced ARF in rats and to evaluate whether neovascularization in the damaged kidney has a role in the therapeutic efficacy of these stem cells in the disease.

## MATERIALS AND METHODS

### Animals

Experiments were performed on Sprague Dawley (SD) rats (220-250 g) purchased from Central Drug Research Institute, Lucknow, India. All animal experimental procedures in this study were performed as per guidelines of the Institutional Animal Ethics Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The protocol was approved by the Animal Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

### Isolation and culture of fKSC

The fKSC were isolated from SD rat fetuses at gestation

day 16. The kidneys were removed from fetuses of 10 pregnant female rats (8-12 fetuses/animal), minced, and digested with 1 mg/mL collagenase type-Ⅳ (Worthington Biochemical, NJ, United States) in serum free α-MEM (Gibco, NY, United States) medium for 40 min at 37 ℃ with intermittent stirring in water bath. After two washes with α-MEM, the digested tissue was cultured at 37 ℃ in a 5% $CO_2$ in 25 $cm^2$ tissue culture flasks (BD, New Jersey, United States) in complete culture medium consisting of α-MEM medium, 2 mg/mL of Glutamax (Gibco, NY, United States), 16.5% fetal bovine serum (Gibco, NY, United States) and bacteriostatic level of penicillin-streptomycin (Gibco, NY, United States). After 48 h of seeding of fetal kidney cells, the culture media containing non-adherent cells were replaced. On day 3 adherent cells were harvested by trypsinization with TrypLE Express (Gibco, NY, United States) and further cultured in complete culture media. The cells of 3rd passage were used for this study.

### Characterization of fKSC

The fKSC were characterized by studying their expression of mesenchymal and renal progenitor markers. The expression of mesenchymal markers on fKSC was studied by flow cytometry. The fKSC at passage 3 were incubated for 30 min with following antibodies: Fluorescein isothiocyanate (FITC) conjugated CD90 (Abcam, MA, United States), CD44 (BD Biosciences, CA, United States), phycoerythrin conjugated CD45 (Abcam, MA, United States), MHC class Ⅱ (Abcam, MA, United States) and with unconjugated CD29 (Abcam, MA, United States), CD73 (BD Biosciences, CA, United States), CD105 (Santa cruz biotechnology, inc. CA, United States). The corresponding FITC-conjugated secondary antibodies (Abcam, MA, United States) were added in CD29, CD73 and CD105 tubes. Isotype-identical antibodies (IgG) served as controls.

Expression of renal progenitor markers *viz*. Wilms tumor1 (Wt1), paired box2 (Pax2), SIX homebox2 (Six2) was studied by immunocytochemistry. The cells were fixed with 4% para-formaldehyde for 1 h at room temperature and incubated overnight at 4 ℃ with following primary antibodies: Wt1, Pax2 (both from Abcam, MA, United States) and Six2 (Santa cruz biotechnology, Inc. CA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies and counter stained with Hoechst dye (Sigma-Aldrich, MO, United States). The fKSC incubated with non-immune serum or IgG in place of primary antibodies were used as negative controls. The images were obtained using Nuance Multispectral Imaging System (CRi Inc., MA, United States).

### In vitro differentiation towards epithelial lineage and angiogenic assay

To induce epithelial differentiation, fKSC ($2 \times 10^4$ cells/well) were cultured on cover slips in 6 well plate in complete culture medium containing 0.1 μmol/L Retinoic acid, 10 ng/mL activin-A and 50 ng/mL Bmp7 (all from R and D Systems, MN, United States). After 7 d, cover slips with induced fKSC were fixed in 4% para-formaldehyde for 1 h at room temperature. The fixed cells were incubated at 4 ℃ overnight with primary antibodies *viz*. cytokeratin (CK)18 and CK19 (Abcam, MA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies for 1 h at room temperature and counter stained with Hoechst dye (Sigma-Aldrich, MO, United States). The fKSC cultured without induction medium were used as negative controls.

The tube-formation assay was performed to assess the angiogenic potential of fKSC. Briefly, fKSC ($2 \times 10^4$ cells/well) were seeded onto the growth factor reduced matrigel (BD Biosciences, CA, United States) coated cover slips in 6 well plate and incubated overnight in standard culture condition with complete medium. The cells were examined by phase contrast microscopy and immunocytochemistry. For immunocytochemistry, cells were fixed with 4% para-formaldehyde for 1 h at room temperature. The fixed cells were incubated at 4 ℃ overnight with primary antibodies *viz*. CD31 (AbD serotech, Oxford, United Kingdom) and Von Willebrand factor (vWF) antibody (Abcam, MA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies for 1 h at room temperature and stained with Hoechst dye. The fKSC cultured without growth factor reduced matrigel were used as negative controls.

### Development of ARF model and fKSC therapy

ARF was induced in male SD rats ($n$ = 36) weighing 200-225g by cisplatin injection as described by Ozyurt *et al*[14]. Briefly, the animals were given a single intra-peritoneal injection of cisplatin (0.5 mg in 1 mL of saline, sigma, United States) at a dose of 7 mg/kg of body weight. A significant increase in blood biochemical parameters and renal damage were observed in rats on day 5 of cisplatin injection. Hence at this time point, rats were randomized into two groups *viz*. fKSC treated group ($n$ = 18) and saline treated group ($n$ = 18) for evaluation of the efficacy of stem cell therapy. In addition, a group of healthy rats ($n$ = 6) was also included in the study as healthy controls to compare renal function and histology of fKSC and saline treated groups.

The fKSC were labeled with PKH26, a red fluorescent cell linker (Sigma-Aldrich, MO, United States) according to manufacturer's protocol. Labeling efficiency of PKH26 with fKSC and cell viability of labeled cells were > 95% and > 97% as revealed by fluorescent microscopy and trypan blue exclusion, respectively. On day 5 of cisplatin injection, a total of $2 \times 10^6$ PKH26 labeled fKSC in 150 μL of saline or 150 μL saline alone were injected intravenously through tail vein in each rat of fKSC treated or saline treated groups, respectively.

Animals were euthanized by barbiturate overdose

(intravenous injection, 150 mg/kg pentobarbital sodium) before fKSC therapy (day 0) and after three days (day 3) and seven days (day 7) of fKSC therapy. Blood samples were collected to determine blood urea nitrogen (BUN) and serum creatinine levels by using commercial Span diagnostic kits with an autoanalyser (BioSytems BTS-330). The kidney tissues were excised to perform the assays described below.

### Histopathological and Immunohistochemical analysis

Ten percent of formalin fixed kidney tissues were cut into 5 μm serial sections and stained with hematoxylin and eosin to evaluate sequential histopathological changes in saline and fKSC treated animals. Quantitative assessment of renal tubular necrosis was performed using the grading scores of Jablonski *et al*[15]. For immunohistochemical analysis, 5 μm thick paraffin sections of kidneys were deparaffinized with xylene and rehydrated in a series of alcohol and water. After rehydration, tissue sections were incubated with primary antibodies *viz*. aquaporin (AQP)1, vWF (both from Abcam, MA, United States) and CD31 (AbD serotech, Oxford, United Kingdom) and proliferating cell nuclear antigen (PCNA; BD Biosciences, CA, United States). After overnight incubation, sections were washed with PBS and incubated with corresponding FITC-conjugated secondary antibodies and then counter stained with Hoechst dye. Images were taken by Nuance Multispectral Imaging System (CRi Inc., MA, United States). To determine the capillary density, CD31 stained capillaries were counted in 10 randomly chosen high-power fields (HPF; 20 μm) using a fluorescent microscope and expressed as capillaries per HPF. To determine the number of proliferating cells in the kidney, PCNA positive nuclei were counted in 10 HPFs per section (20 μm) in the cortico-medullary area under a Nuance Multispectral Imaging System (CRi Inc., MA, United States) and the proliferative index was expressed as average number of $PCNA^+$ cells /HPF.

### dUTP nick-end labeling

Apoptotic scores in kidney tissue sections were measured by the terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) as per manufacturer's instruction. Briefly, kidney sections were deparaffinized, rehydrated, and digested with proteinase K and labeled with TUNEL reaction mixture for 60 min at 37 ℃. TUNEL positive nuclei were counted in 10 HPF per section in the cortico-medullary area at 20 μm magnification under a Nuance Multispectral Imaging System (CRi Inc., MA, United States) and the apoptotic index was expressed as average number of $TUNEL^+$ cells /HPF.

### Western blot

Expression of angiogenic signaling molecules in the kidneys of rats was evaluated by western blotting on day 3 after fKSC therapy. The kidney tissues of animals were homogenized in RIPA buffer containing 1 mmol/L phenylmethanesulphonyl fluoride and 1% protease inhibitor cocktail (Sigma-Aldrich, MO, United States). Kidney tissue homogenate was centrifuged at 10000 *g* for 10 min and the supernatant was stored in -80 ℃. 40 mg proteins were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA for 1 h at room temperature and then incubated at 4 ℃ overnight against primary antibodies viz. vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1α and endothelial nitric oxide synthase (eNOS) (all from Abcam, MA, United States). β-Actin antibody (Abcam, MA, United States) was used as a loading control. Primary antibodies were detected by corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies using super signal west pico chemiluminescent substrate (Thermo scientific, IL, United States). The bands were quantified by densitometry using the Quantity One software (Bio-Rad, CA, United States).

### Animal care and use

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

### Statistical analysis

Values were expressed as mean ± standard error (SE). The statistically significant differences between groups were analyzed by one way-analysis of variance (ANOVA) with Bonferroni multiple-comparison post hoc test. Statistical analysis was performed using GraphPad Prism Software Version 5 (GraphPad, San Diego, CA) and a $P$ value of $< 0.05$ was considered statistically significant.

## RESULTS

### Expression of mesenchymal and renal progenitor markers by fKSC

The fKSC exhibited spindle-shaped and polygonal morphology in culture (Figure 1A). Flow cytometry analysis showed that fKSC expressed CD29 (37.59%), CD44 (32.76%), CD73 (51.72%), CD90 (68.71%) and CD105 (37.88%), whereas the expression of CD45 and MHC class Ⅱ was < 5% (Figure 1B). Immunocytochemistry revealed that fKSC also expressed renal progenitor markers *viz*. Wt1, Pax2 and Six2 (Figure 1C).

### In vitro epithelial differentiation and angiogenic potential of fKSC

On treatment with specific induction medium, fKSC

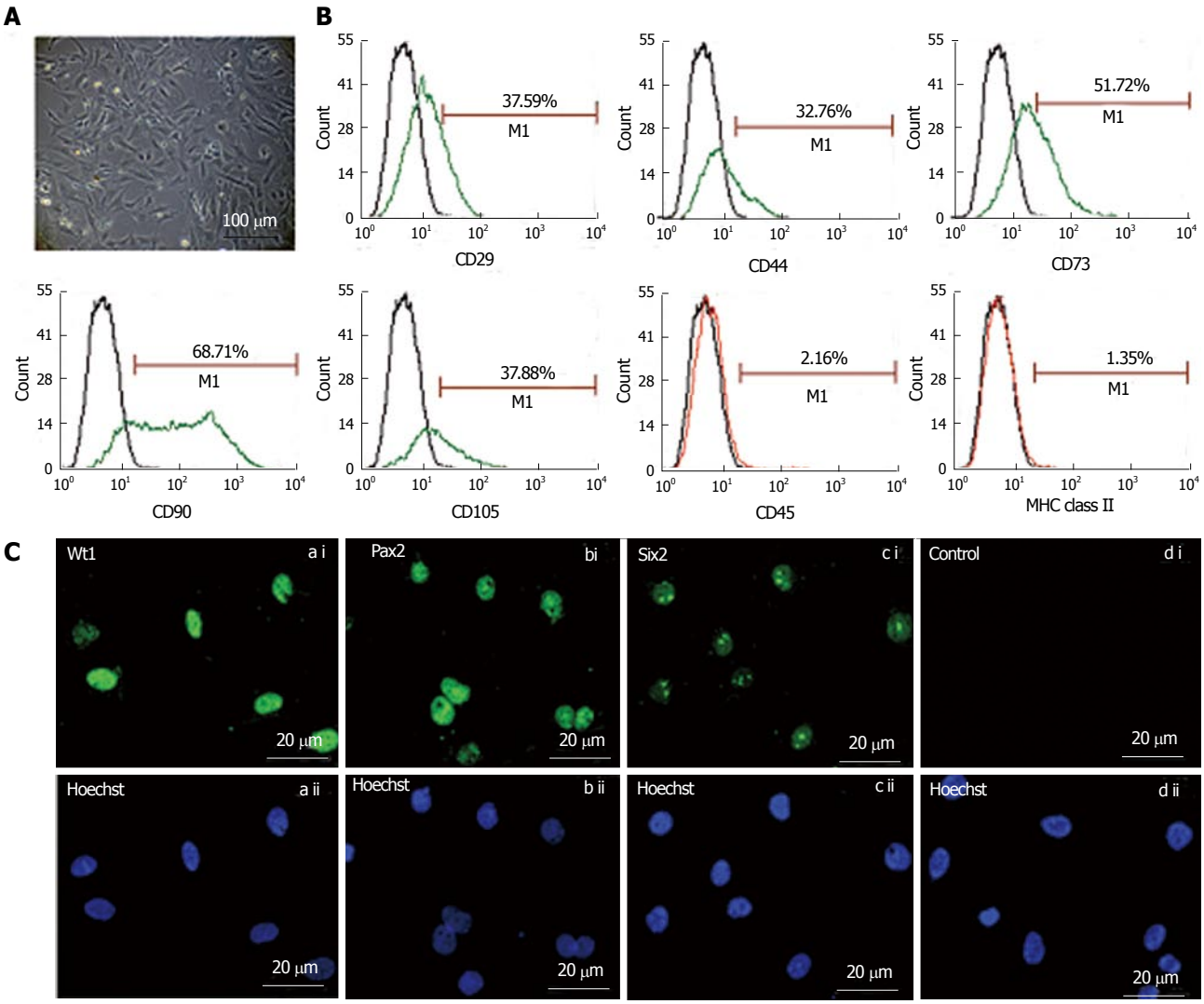


**Figure 1 Morphology (A) and characterization of fetal kidney stem cells (B and C).** A: Representative photomicrograph (Scale bars indicate 100 μm) showing spindle-shaped and polygonal morphology of fetal kidney stem cells (fKSC); B: Phenotypic characterization of fKSC by flow cytometry showing expression of CD29, CD44, CD73, CD90, CD105, CD45, and MHC class II (green or red lines, detected with FITC - or phycoerythrin-conjugated antibodies, respectively) with isotype controls (black lines); C: Representative immunoflourescent photomicrographs (Scale bars indicate 20 μm) showing expression of renal progenitor markers *viz.* Wt1, Pax2 and Six2 on

exhibited differentiation ability into epithelial cells as demonstrated by expression of CK18 and CK19 by immunocytochemistry (Figures 2A and B). When grown on matrigel, fKSC exhibited capillary-like tubular structures assessed by phase contrast microscopy (Figure 2C) and expressed endothelial markers *viz.*, CD31 and vWF by immunocytochemistry (Figure 2D and E).

### *Improvement of renal function following fKSC therapy in ARF rats*

On the 5th day of cisplatin injection, the rats exhibited a significant increase in levels of both BUN and serum creatinine as compared to healthy controls ($P < 0.05$). The same day (day 0 of therapy), the rats were randomized to receive fKSC therapy or saline alone. On day 3 of therapy, fKSC treated group had significantly lower levels of these blood biochemical parameters as compared to saline treated group ($P < 0.05$), but both groups had significantly higher levels as compared to healthy controls ($P < 0.05$). On day 7 of therapy, the serum creatinine and BUN levels in the fKSC treated group were comparable to healthy controls but they were still significantly higher in the saline treated group in comparison to healthy controls ($P < 0.05$) (Figure 3A and B).

### *Improvement of renal histology following fKSC therapy in ARF rats*

The kidneys of ARF rats on the 5th day of cisplatin injection showed severe tubular necrosis, hyaline cast formation, loss of brush border in the proximal renal tubules and tubular dilatation (Figure 4B). On day 3 of fKSC therapy, the kidneys showed a significant attenuation of tubular injury as compared to saline treated rats (Figure 4C and D) and Jablonski grading score revealed significantly lower necrosis in the kidneys of fKSC treated as compared to saline treated

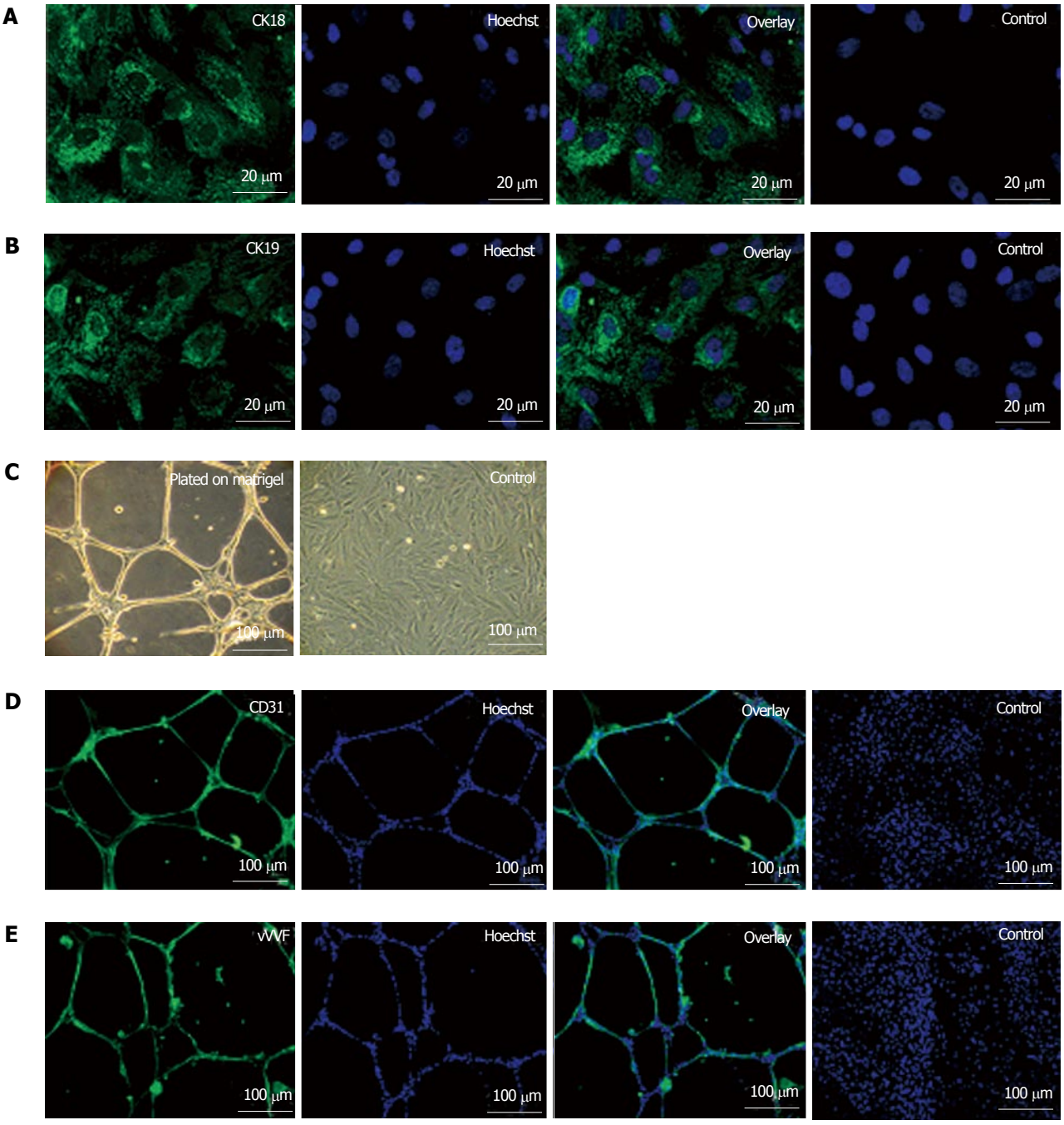


**Figure 2 Epithelial differentiation (A, B) and angiogenic potential (C-E) of fetal kidney stem cells.** A: Representative photomicrograph (Scale bars indicate 20 μm) showing CK18 expression, Hoechst, overlay and untreated control cells with only Hoechst; B: Representative photomicrograph (Scale bars indicate 20 μm) showing CK19 expression, Hoechst, overlay and untreated control cells with only Hoechst; C: Representative photomicrographs (Scale bars indicate 100 μm) showing tubule like structure formation by fetal kidney stem cells (fKSC) cultured on matrigel and without matrigel as control; D: Representative immunoflourescent photomicrographs (Scale bars indicate 100 μm) showing CD31 expression, Hoechst, overlay and fKSC cultured without matrigel as controls with only Hoechst; E: Representative immunoflourescent photomicrographs (Scale bars indicate 100 μm) of fKSC cultured on matrigel showing Von Willebrand factor expression, Hoechst, overlay and fKSC cultured without matrigel as controls with only Hoechst.

rats (2.33 ± 0.33 *vs* 3.50 ± 0.34, $P < 0.05$, Figure 4G). On day 7 of therapy, the kidneys of fKSC treated ARF rats showed disappearance of necrotic cells with almost normal architecture of tubules, whereas those of saline treated ARF rats still had necrotic tubular cells and hyaline casts (Figure 4E and F) and there was also a significant difference in Jablonski grading score between the fKSC treated and saline treated groups (0.83 ± 0.16 *vs* 2.00 ± 0.25, $P < 0.05$, Figure 4G).

### *Engraftment of infused fKSC in renal tubules*

To study their engraftment, PKH26 labeled fKSC were evaluated in rat kidneys on day 7 of therapy. The fKSC were observed to engraft preferentially in renal tubules and capillaries stained with tubular epithelial marker AQP1 and endothelial marker vWF, respectively (Figure

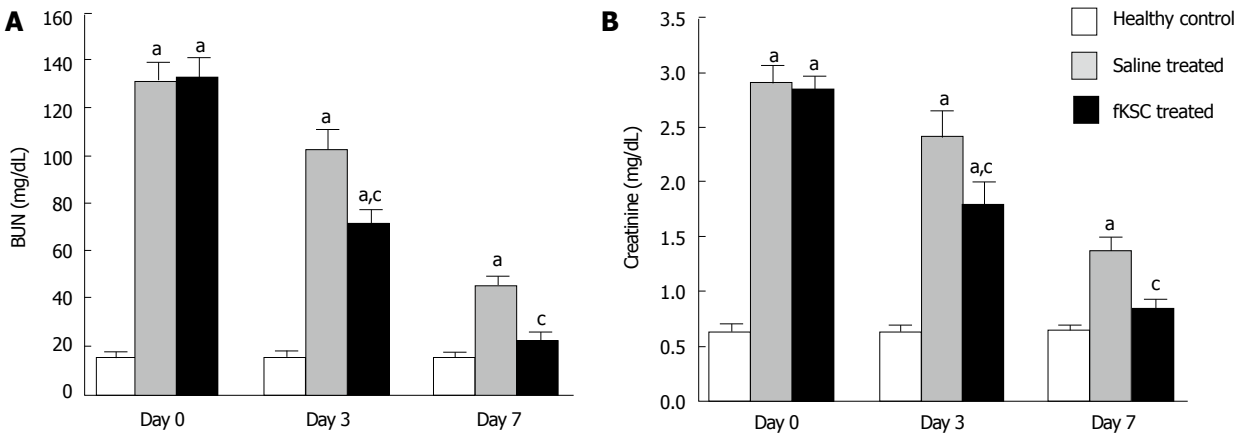


**Figure 3 Effect of fetal kidney stem cells therapy on renal function in rats with cisplatin induced acute renal failure.** The changes in the levels of (A) blood urea nitrogen (BUN) and (B) serum creatinine in healthy control, saline treated and fetal kidney stem cells (fKSC) treated groups on day 0, 3 and 7 after fKSC therapy. Values expressed as Mean ± SE. $^{a}P < 0.05$ for saline treated vs healthy control group; $^{c}P < 0.05$ for fKSC *vs* saline treated group.

5).

### *Administration of fKSC reduces apoptosis and promotes proliferation of renal tissues*

On day 3 of therapy, the TUNEL-positive cells in the kidneys of fKSC treated rats were significantly lower than those in the saline treated rats ($P < 0.05$). On day 7, their numbers further decreased significantly in fKSC treated rats as compared to those in the saline treated rats ($P < 0.05$; Figure 6A). On days 3 and 7 of therapy, the number of PCNA-positive cells in the kidneys of fKSC treated rats were significantly higher as compared to those in the saline treated rats ($P < 0.05$, Figure 6B).

### *Administration of fKSC promotes renal angiogenesis*

On day 3 of therapy, the morphometric analysis of CD31 labeled peritubular capillaries showed a significantly higher capillary density in the kidneys of fKSC treated than that in saline treated rats (13.30 ± 1.54 *vs* 7.10 ± 1.29, capillaries/HPF, $P < 0.05$). On day 7 of therapy, the capillary density in the kidneys of fKSC treated rats was further significantly increased than that in saline treated rats (21.10 ± 1.46 *vs* 15.00 ± 1.30, capillaries/HPF, $P < 0.05$; Figure 7).

To determine the early angiogenic effect of fKSC, the protein expression of angiogenic signaling molecules were studied on day 3 of fKSC therapy. The expressions of HIF-1$\alpha$, VEGF and eNOS in the kidneys of fKSC treated rats were significantly higher as compared to saline treated rats ($P < 0.05$; Figure 8).

## DISCUSSION

The present study demonstrates that fKSC expressed mesenchymal as well as renal progenitor markers and exhibited the formation of CD31 and vWF expressing capillary-like structures and differentiation into CK18 and CK19 positive epithelial cells *in vitro*. The administration of fKSC in rats with cisplatin induced ARF resulted in rapid improvement in renal function and histology. The infused fKSC were observed to engraft in renal tubules and promote proliferation and reduce apoptosis of renal tubular cells. In addition, the kidneys of fKSC treated rats exhibited increased angiogenesis and up-regulation of angiogenic signaling molecules. To our knowledge this is the first study showing therapeutic efficacy of *in vitro* expanded fKSC in cisplatin induced ARF model and role of angiogenesis in renal regeneration by these stem cells.

We have recently observed that fKSC have maximal growth at a seeding density of 1000 cells/cm$^2$, population doubling time of approximately 34 h and normal karyotype up to 3$^{rd}$ passage. In addition, these cells successfully differentiated into cells of all the three germ layers (communicated elsewhere). In the present study, we have observed that fKSC express mesenchymal (CD29, CD44, CD73, CD90 and CD105) as well as renal progenitor markers (Wt1, Pax2 and Six2). The Pax2 and Wt1 genes cross-regulate expression of each other during kidney development[16]. However, we have observed a high expression of these markers in fKSC. A plausible explanation of this could be that a significant proportion of fKSC are a population of stem cells of metanephric mesenchyme which have been shown to express both Pax2 and Wt1 transcription factors[13,17,18]. Similar to our observation high expression of Pax2 and Wt1 has recently been shown in human fetal kidney progenitors as well as human embryonal cells derived nephron progenitors[19]. The fKSC could be induced to differentiate *in vitro* into CK18 and CK19 positive epithelial cells. In addition, they exhibited an angiogenic potential as shown by the formation of CD31 and vWF positive capillary-like tubular structures when plated on matrigel. Two previous studies have shown that stem cells derived from whole fetal kidneys express mesenchymal and renal progenitor markers, but they have not studied their *in vitro* angiogenic potential and differentiation into epithelial cells[20,21]. However, corroborating with our data, one study has shown that human embryonic kidney derived CD24$^+$ CD133$^+$ stem cells exhibit *in vitro* differentiation into epithelial

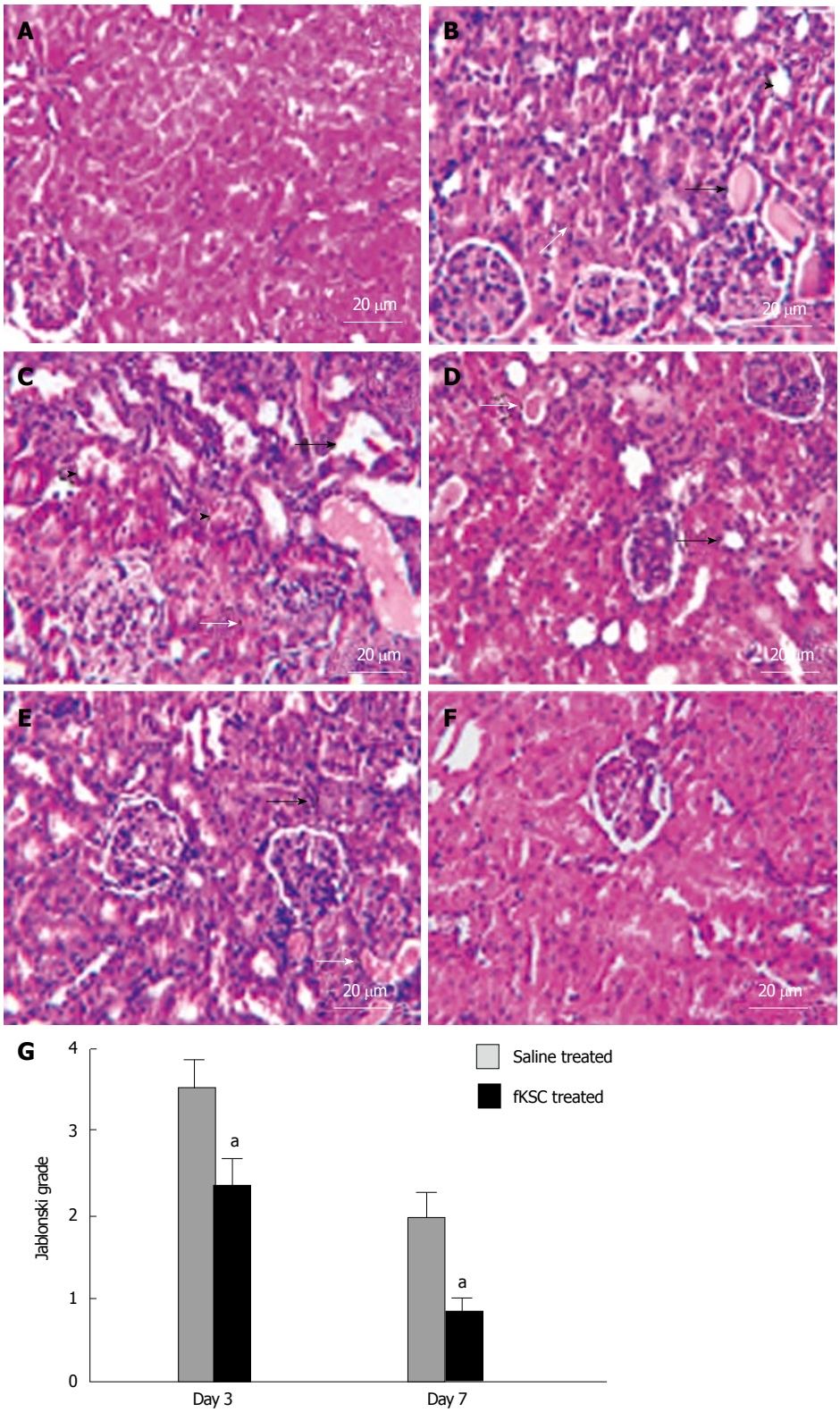


**Figure 4 Fetal kidney stem cells attenuate renal structural damage in rats with cisplatin induced acute renal failure (Scale bars indicate 20 μm).** A: Kidney section of healthy control rats showing the normal architecture of tubules and glomeruli; B: Kidney section after 5 d of cisplatin treatment showing severe tubular necrosis (white arrow), hyaline cast formation (black arrow) and tubular dilatation (black arrow head); C: Kidney section of saline treated rats on day 3 of therapy showing severe tubular necrosis (white arrow), tubular vacuolization (black arrow) and tubular swelling and obstruction (black arrow head) with cell debris on day 3 after therapy; D: Kidney section of fetal kidney stem cells (fKSC) treated rats on day 3 of therapy showing attenuation of tubular injury with mild tubular dilatation (black arrow) and few hyaline casts inside the lumen (white arrow); E: Kidney section of saline treated rats on day 7 of therapy showing few necrotic tubular cells (black arrow) and luminal hyaline casts (white arrow); F: Kidney section of fKSC treated rats on day 7 of therapy fKSC showing almost normal architecture of tubules; G: Jablonski grading score of histological assessment of tubular necrosis in saline and fKSC treated kidneys on day 3 and day 7. Values expressed as mean ± SE. $^{a}P < 0.05$ for fKSC *vs* saline treated group.

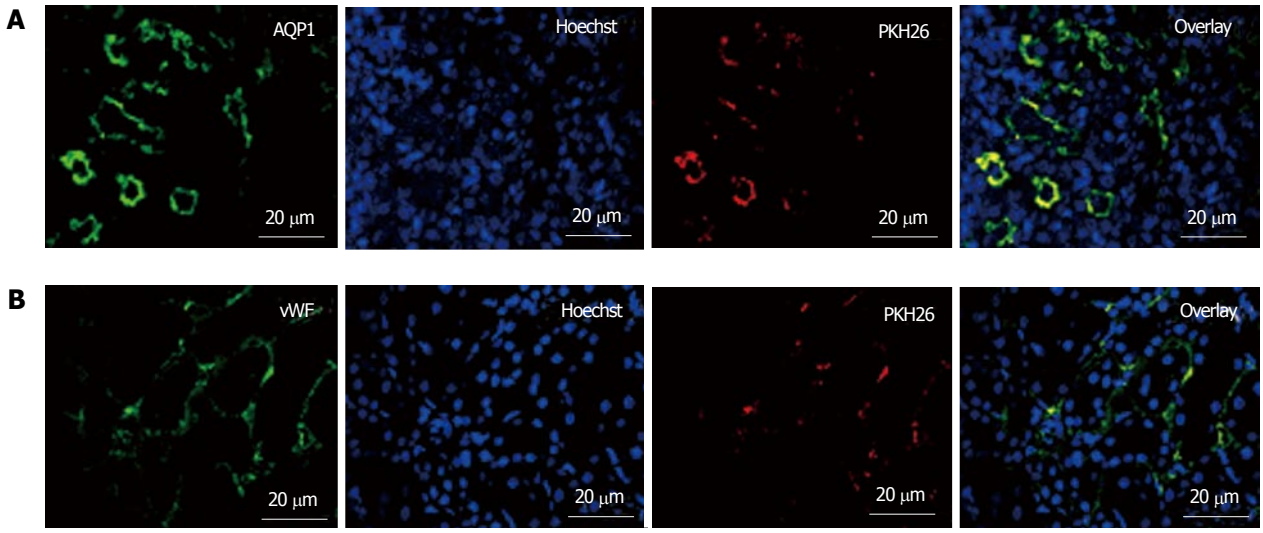


**Figure 5** ***In vivo*** **homing and engraftment of administered fetal kidney stem cells in renal tubules**. Representative immunofluorescent photomicrographs (Scale bars indicate 20 μm) showing engraftment of administered fetal kidney stem cells (fKSC) in renal tubules at day 7 of therapy, and expression of tubular epithelial and endothelial markers aquaporin (AQP)1 (A) and Von Willebrand factor (vWF) (B) respectively, with corresponding Hoechst, PKH26 labeled administered fKSC in injured kidney tissue respectively.

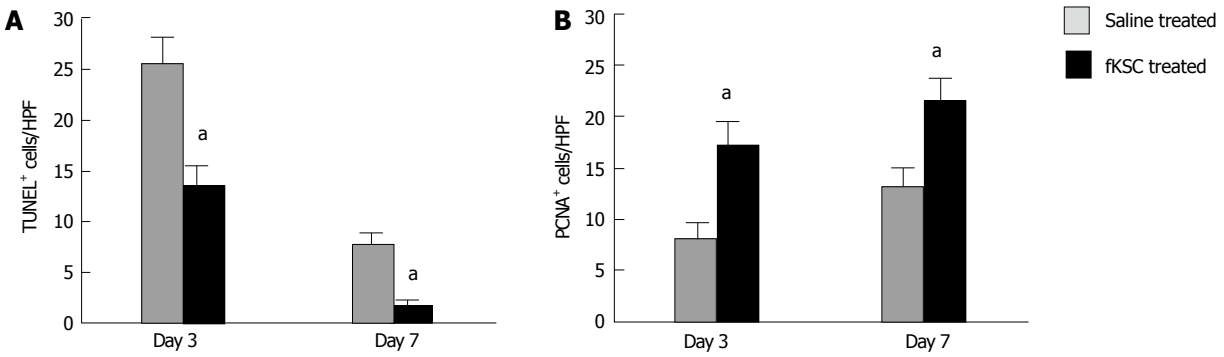


**Figure 6 Effects of fetal kidney stem cells on apoptosis and proliferation of tubular cells in cisplatin injured kidney.** A: Quantification of dUTP nick-end labeling (TUNEL)-positive cells in kidney sections of saline and fetal kidney stem cells (fKSC) treated groups at day 3 and 7 after fKSC therapy; B: Quantification of proliferating cell nuclear antigen (PCNA)-positive cells in kidney sections of saline and fKSC treated groups at day 3 and 7 after fKSC therapy. Values expressed mean ± SE. $^{a}P < 0.05$ for fKSC *vs* saline treated group. HPF: High-power field.

and endothelial lineages[22]. We did not evaluate the markers of other renal progenitors such as endothelial and epithelial ones on fKSC which may be a limitation of the present study.

To investigate their therapeutic effect in ARF, we infused intravenously PKH26 labeled fKSC in cisplatin induced rat model of ARF. On day 3 of therapy, we observed a significant improvement in the renal function and histology and on day 7 these parameters became comparable to healthy control rats indicating almost complete recovery. Similar to our data, culture expanded $CD24^+$ $CD133^+$ stem cells from human embryonic kidney have been shown to mediate a complete functional and structural recovery of glycerol induced ARF in SCID mice[22]. We observed that PKH26 labeled fKSC engraft in the damaged areas of the kidney and express vWF and AQP1 showing the integration of infused fKSC into damaged renal vasculature and tubules, respectively. It is difficult to distinguish whether vWF and AQP1 are expressed by endothelial and epithelial cells differentiated from infused fKSC or these markers represent fusion of administered fKSC with damaged kidney cells in the areas of engraftment. Similar to our observation, administration of human embryonic kidney derived $CD24^+$ $CD133^+$ stem cells in SCID mice with glycerol induced ARF and embryonic stem cell derived mesenchymal like progenitors in rats with cisplatin induced ARF have been shown to engraft into damaged renal tubules and vasculature of kidney[22,23].

The kidneys of fKSC treated ARF rats were observed to have a higher capillary density correlating with their structural and functional improvement as compared to saline treated ARF rats. In addition, on day 3 of fKSC therapy, there was an increased expression of HIF-1α and its downstream signaling molecules VEGF and eNOS in the kidneys of fKSC treated rats in comparison to saline treated rats, showing a role of early angiogenesis in the renal regeneration. It has been reported that HIF-1α also activates expression of

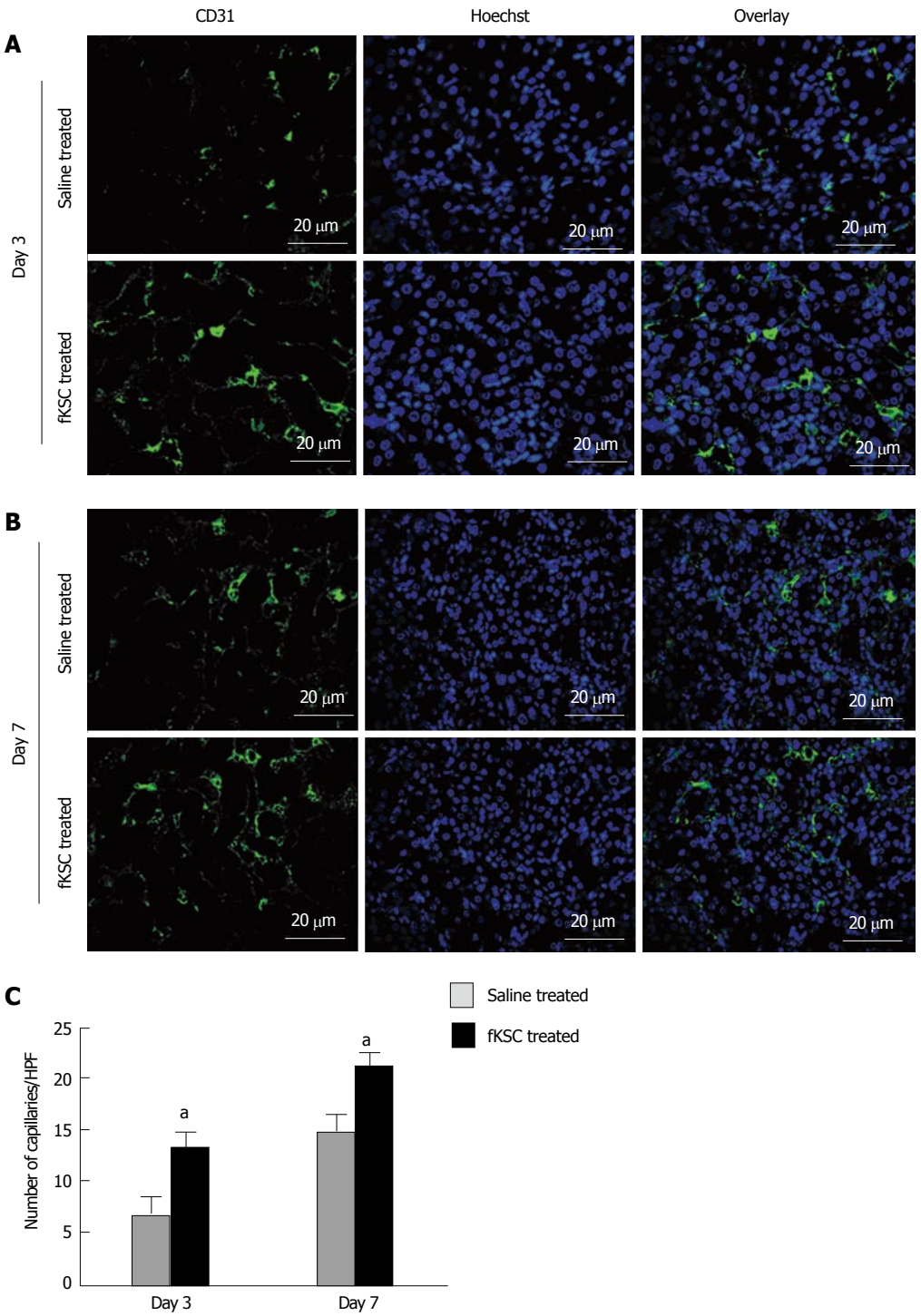


**Figure 7 Fetal kidney stem cells accelerate angiogenesis in rats with cisplatin induced acute renal failure.** Representative immunoflourescent photomicrographs (Scale bars indicate 20 μm) showing perivascular capillaries stained with CD31 in saline treated and fetal kidney stem cells (fKSC) treated groups on day 3 (A) and on day 7 (B) after fKSC therapy. C: Quantification of perivascular capillaries stained with CD31, expressed as capillaries per HPF, in saline and fKSC treated groups. Values expressed Mean ± SE. $^{a}P < 0.05$ for fKSC *vs* saline treated group. HPF: High-power field.

Wt1, which in turn directly up-regulates VEGF and thus acts as an important regulator of angiogenesis[24-27]. The fKSC that were infused in ARF rats were already expressing Wt1, so we did not study its expression in the kidney tissues but as fKSC therapy up-regulated HIF-1α, it is likely that kidney tissues also have an up-regulation of Wt1. However, since the angiogenic role of Wt1 has mostly been reported in tumors, it is not known whether Wt1 also has a similar role in normal tissues like kidney. Corroborating with our data, it has been shown that acetazolamide administration in mice with ischemic kidney results in up-regulation of angiogenic molecules HIF-1α and eNOS in kidney tissue along with reduction in the apoptosis of renal tubular cells, increase in renal blood flow and improvement in renal function and histology[28]. Similarly, the

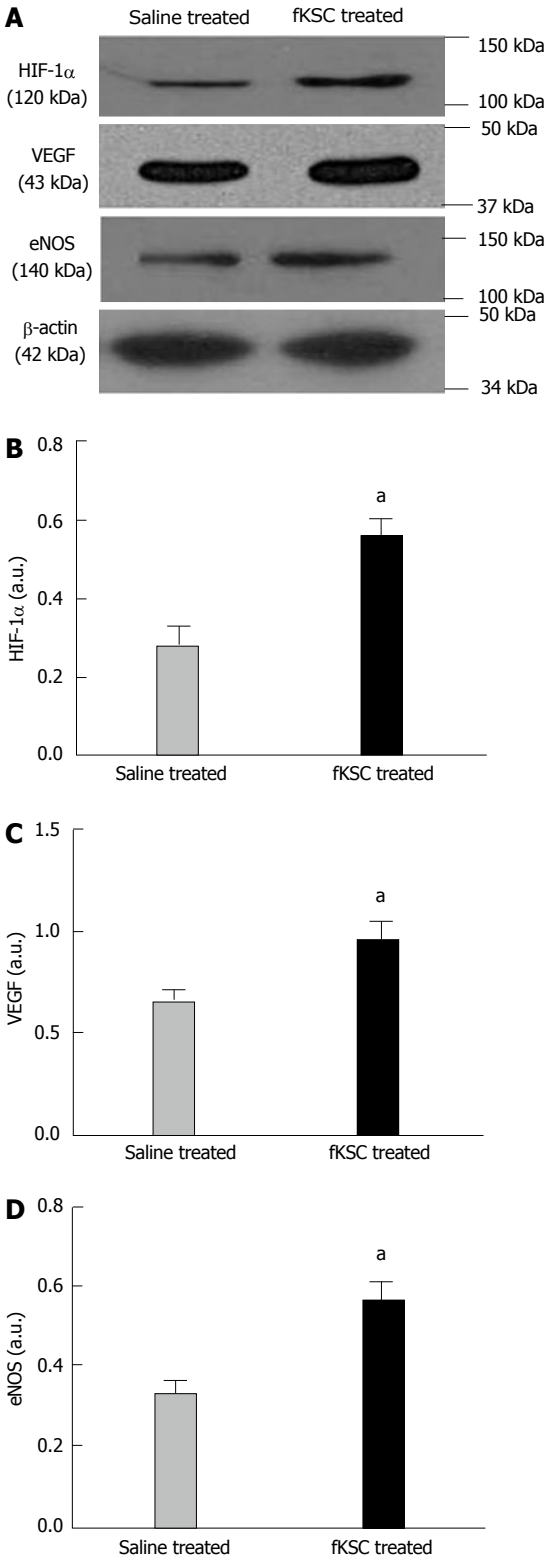


**Figure 8 Early angiogenic effect of administered fetal kidney stem cells in cisplatin injured kidney.** Representative immunoblots showing the expression of hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in saline and fetal kidney stem cells (fKSC) treated groups on day 3 after fKSC therapy (A-D). Bar diagrams showing densitometric quantification of the expression of HIF-1α (B), VEGF (C) and eNOS (D). Comparative gene expression ratio was calculated by referring each gene to β-actin as an internal control. Densitometric analysis applied for comparison of relative protein expression and represented in densitometric arbitrary units (a. u.). Values expressed mean ± SE. $^{a}P < 0.05$ for fKSC *vs* saline treated group.

VEGF has been shown to promote regeneration of cisplatin induced injury of renal tubular epithelial cells *in vitro* by inducing proliferation and reducing their apoptosis. In addition, administration of VEGF modified embryonic mesenchymal stem cells (MSC) in cisplatin induced ARF in rodents has been shown to reduce renal apoptosis and promote microcirculation and cell proliferation in the damaged kidney[29]. There is no previous study showing angiogenic role of fKSC in ARF but in agreement with our study, it has been shown that a clonal cell line of MSC derived from kidney of adult Tie-2 green fluorescent protein transgenic mice exhibit *in vivo* angiogenesis and tubular regeneration by increasing epithelial proliferation and inhibiting apoptosis when administered in mouse model of renal ischemia[30]. These studies suggest that the improved capillary density, proliferation and reduced apoptosis in renal tissue observed by us following fKSC therapy may be mediated by the up-regulation of VEGF, HIF-1α and eNOS, and thus these molecules could play an important role in renal regeneration.

In conclusion, the present study shows that fKSC express mesenchymal and renal progenitor markers, exhibit an *in vitro* angiogenic potential and ability to differentiate into cells of renal epithelial lineage. The administration of fKSC in cisplatin induced ARF results in rapid recovery of renal function and histology, and promotes renal angiogenesis. Further studies on fKSC mediated renal angiogenesis and regeneration may lead to the development of novel pharmacological therapies for ARF.

## ACKNOWLEDGMENTS

We would like to extend our sincere thanks to Dr. Rituraj Konwar, Scientist, Endocrinology Division, CSIR-Central Drug Research Institute, Lucknow, India and Dr. SK Mandal, Consultant Statistician, Center for Bio-Medical Research Lucknow, India for extending laboratory facilities to perform the Flow cytometry and review of statistical analysis, respectively.

## COMMENTS

### *Background*

Tissue specific fetal stem cells represent the most suitable stem cell type for tissue regeneration/repair due to their inherent ability of tissue specific differentiation. Thus, stem cells derived from the fetal kidney may be a novel cell type for renal regenerative therapy. The epithelial and microvascular damage are the main events in acute renal failure (ARF) and this led us to evaluate whether fetal kidney stem cells (fKSC) mediate their therapeutic effect by augmenting renal angiogenesis in cisplatin induced ARF in rats.

### *Research frontiers*

The authors had demonstrated in the present study that fKSC differentiate into cells of epithelial lineage and exhibit angiogenic potential *in vitro*. Following infusion in ARF rats, they engraft in renal tubules and rapidly improve renal function and histology along with increased angiogenesis in the kidney tissues. It is thus logical to investigate whether induction of renal angiogenesis by fKSC is an important mechanism of renal regeneration and whether it is mediated by trans-differentiation of infused fKSC or their paracrine effect on resident kidney

stem cells.

### Innovations and breakthroughs

To the best of our knowledge, this is the first study showing therapeutic efficacy of *in vitro* expanded fKSC in ARF model and their *in vitro* and *in vivo* angiogenic potential suggesting that induction of renal angiogenesis may be an important therapeutic mechanism of these stem cells.

### Applications

The fKSC represent primitive renal progenitors expressing mesenchymal as well as renal progenitor markers and treatment of ARF rats with these stem cells results in rapid recovery in renal function and histology leading to their normalization. Further studies using human fKSC or human embryonic stem cell derived renal progenitors with similar characteristics may lead to the development of clinical therapy for ARF.

### Terminology

The fKSC represent primitive renal progenitors and express mesenchymal as well as renal progenitor markers. They can be culture expanded to generate a requisite number of cells for therapeutic dose. They improve renal function and histology and lead to their normalization by inducing renal angiogenesis. It needs to be studied whether the angiogenesis observed in the kidney tissues in the fKSC treated rats is due to trans-differentiation of infused stem cells or by stimulation of resident endothelial cells or both.

### Peer-review

It is a very interesting article about a possible protective role of fetal kidney stem cells in experimental model of acute renal failure.

## REFERENCES


1 **Schrier RW**, Wang W, Poole B, Mitra A. Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J Clin Invest* 2004; **114**: 5-14 [PMID: 15232604 DOI: 10.1172/JCI22353]

2 **Coca SG**, Singanamala S, Parikh CR. Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney Int* 2012; **81**: 442-448 [PMID: 22113526 DOI: 10.1038/ki.2011.379]

3 **Cirio MC**, de Groh ED, de Caestecker MP, Davidson AJ, Hukriede NA. Kidney regeneration: common themes from the embryo to the adult. *Pediatr Nephrol* 2014; **29**: 553-564 [PMID: 24005792 DOI: 10.1007/s00467-013-2597-2]

4 **Patschan D**, Patschan S, Müller GA. Microvasculopathy in ischemic acute kidney injury: consequences and therapeutic implications. *Panminerva Med* 2012; **54**: 45-52 [PMID: 22278116]

5 **Hörbelt M**, Lee SY, Mang HE, Knipe NL, Sado Y, Kribben A, Sutton TA. Acute and chronic microvascular alterations in a mouse model of ischemic acute kidney injury. *Am J Physiol Renal Physiol* 2007; **293**: F688-F695 [PMID: 17626153 DOI: 10.1152/ajprenal.00452.2006]

6 **Sutton TA**, Fisher CJ, Molitoris BA. Microvascular endothelial injury and dysfunction during ischemic acute renal failure. *Kidney Int* 2002; **62**: 1539-1549 [PMID: 12371954 DOI: 10.1046/j.1523-1755.2002.00631.x]

7 **Basile DP**. Rarefaction of peritubular capillaries following ischemic acute renal failure: a potential factor predisposing to progressive nephropathy. *Curr Opin Nephrol Hypertens* 2004; **13**: 1-7 [PMID: 15090853 DOI: 10.1097/00041552-200401000-00001]

8 **Chade AR**. Renal vascular structure and rarefaction. *Compr Physiol* 2013; **3**: 817-831 [PMID: 23720331 DOI: 10.1002/cphy.c120012]

9 **Morigi M**, De Coppi P. Cell therapy for kidney injury: different options and mechanisms--mesenchymal and amniotic fluid stem cells. *Nephron Exp Nephrol* 2014; **126**: 59 [PMID: 24854642 DOI: 10.1159/000360667]

10 **Harari-Steinberg O**, Pleniceanu O, Dekel B. Selecting the optimal cell for kidney regeneration: fetal, adult or reprogrammed stem cells. *Organogenesis* 2011; **7**: 123-134 [PMID: 21519195 DOI: 10.4161/org.7.2.15783]

11 **Dekel B**, Reisner Y. Embryonic committed stem cells as a solution to kidney donor shortage. *Expert Opin Biol Ther* 2004; **4**: 443-454 [PMID: 15102595 DOI: 10.1517/14712598.4.4.443]

12 **Dekel B**, Burakova T, Arditti FD, Reich-Zeliger S, Milstein O, Aviel-Ronen S, Rechavi G, Friedman N, Kaminski N, Passwell JH, Reisner Y. Human and porcine early kidney precursors as a new source for transplantation. *Nat Med* 2003; **9**: 53-60 [PMID: 12496960 DOI: 10.1038/nm812]

13 **Oliver JA**, Barasch J, Yang J, Herzlinger D, Al-Awqati Q. Metanephric mesenchyme contains embryonic renal stem cells. *Am J Physiol Renal Physiol* 2002; **283**: F799-F809 [PMID: 12217872 DOI: 10.1152/ajprenal.00375.2001]

14 **Ozyurt H**, Yildirim Z, Kotuk M, Yilmaz HR, Yağmurca M, Iraz M, Söğüt S, Gergerlioglu S. Cisplatin-induced acute renal failure is ameliorated by erdosteine in a dose-dependent manner. *J Appl Toxicol* 2004; **24**: 269-275 [PMID: 15300714 DOI: 10.1002/jat.983]

15 **Jablonski P**, Howden BO, Rae DA, Birrell CS, Marshall VC, Tange J. An experimental model for assessment of renal recovery from warm ischemia. *Transplantation* 1983; **35**: 198-204 [PMID: 6340272 DOI: 10.1097/00007890-198303000-00002]

16 **Eccles MR**, He S, Legge M, Kumar R, Fox J, Zhou C, French M, Tsai RW. PAX genes in development and disease: the role of PAX2 in urogenital tract development. *Int J Dev Biol* 2002; **46**: 535-544 [PMID: 12141441]

17 **Metsuyanim S**, Harari-Steinberg O, Buzhor E, Omer D, Pode-Shakked N, Ben-Hur H, Halperin R, Schneider D, Dekel B. Expression of stem cell markers in the human fetal kidney. *PLoS One* 2009; **4**: e6709 [PMID: 19696931 DOI: 10.1371/journal.pone.0006709]

18 **Yu CT**, Tang K, Suh JM, Jiang R, Tsai SY, Tsai MJ. COUP-TFII is essential for metanephric mesenchyme formation and kidney precursor cell survival. *Development* 2012; **139**: 2330-2339 [PMID: 22669823 DOI: 10.1242/dev.076299]

19 **Kang M**, Han YM. Differentiation of human pluripotent stem cells into nephron progenitor cells in a serum and feeder free system. *PLoS One* 2014; **9**: e94888 [PMID: 24728509 DOI: 10.1371/journal.pone.0094888]

20 **Kim SS**, Gwak SJ, Han J, Park HJ, Park MH, Song KW, Cho SW, Rhee YH, Chung HM, Kim BS. Kidney tissue reconstruction by fetal kidney cell transplantation: effect of gestation stage of fetal kidney cells. *Stem Cells* 2007; **25**: 1393-1401 [PMID: 17548530 DOI: 10.1634/stemcells.2006-0183]

21 **Kim SS**, Park HJ, Han J, Gwak SJ, Park MH, Song KW, Rhee YH, Min Chung H, Kim BS. Improvement of kidney failure with fetal kidney precursor cell transplantation. *Transplantation* 2007; **83**: 1249-1258 [PMID: 17496543 DOI: 10.1097/01.tp.0000261712.93299.a6]

22 **Lazzeri E**, Crescioli C, Ronconi E, Mazzinghi B, Sagrinati C, Netti GS, Angelotti ML, Parente E, Ballerini L, Cosmi L, Maggi L, Gesualdo L, Rotondi M, Annunziato F, Maggi E, Lasagni L, Serio M, Romagnani S, Vannelli GB, Romagnani P. Regenerative potential of embryonic renal multipotent progenitors in acute renal failure. *J Am Soc Nephrol* 2007; **18**: 3128-3138 [PMID: 17978305 DOI: 10.1681/ASN.2007020210]

23 **Luo J**, Zhao X, Tan Z, Su Z, Meng F, Zhang M. Mesenchymal-like progenitors derived from human embryonic stem cells promote recovery from acute kidney injury via paracrine actions. *Cytotherapy* 2013; **15**: 649-662 [PMID: 23415919 DOI: 10.1016/j.jcyt.2013.01.009]

24 **Wagner KD**, Wagner N, Wellmann S, Schley G, Bondke A, Theres H, Scholz H. Oxygen-regulated expression of the Wilms' tumor suppressor Wt1 involves hypoxia-inducible factor-1 (HIF-1). *FASEB J* 2003; **17**: 1364-1366 [PMID: 12738801]

25 **McCarty G**, Awad O, Loeb DM. WT1 protein directly regulates expression of vascular endothelial growth factor and is a mediator of tumor response to hypoxia. *J Biol Chem* 2011; **286**: 43634-43643 [PMID: 22030397 DOI: 10.1074/jbc.M111.310128]

26 **Katuri V**, Gerber S, Qiu X, McCarty G, Goldstein SD, Hammers H, Montgomery E, Chen AR, Loeb DM. WT1 regulates angiogenesis in Ewing Sarcoma. *Oncotarget* 2014; **5**: 2436-2449 [PMID: 24810959]

27 **Wagner KD**, Cherfils-Vicini J, Hosen N, Hohenstein P, Gilson E, Hastie ND, Michiels JF, Wagner N. The Wilms' tumour suppressor Wt1 is a major regulator of tumour angiogenesis and progression. *Nat Commun* 2014; **5**: 5852 [PMID: 25510679 DOI: 10.1038/

ncomms6852]

28 **An Y**, Zhang JZ, Han J, Yang HP, Tie L, Yang XY, Xiaokaiti Y, Pan Y, Li XJ. Hypoxia-inducible factor-1α dependent pathways mediate the renoprotective role of acetazolamide against renal ischemia-reperfusion injury. *Cell Physiol Biochem* 2013; **32**: 1151-1166 [PMID: 24335166 DOI: 10.1159/000354515]

29 **Yuan L**, Wu MJ, Sun HY, Xiong J, Zhang Y, Liu CY, Fu LL, Liu DM, Liu HQ, Mei CL. VEGF-modified human embryonic mesenchymal stem cell implantation enhances protection against cisplatin-induced acute kidney injury. *Am J Physiol Renal Physiol* 2011; **300**: F207-F218 [PMID: 20943766 DOI: 10.1152/ajprenal.00073.2010]

30 **Chen J**, Park HC, Addabbo F, Ni J, Pelger E, Li H, Plotkin M, Goligorsky MS. Kidney-derived mesenchymal stem cells contribute to vasculogenesis, angiogenesis and endothelial repair. *Kidney Int* 2008; **74**: 879-889 [PMID: 18596729 DOI: 10.1038/ki.2008.304]

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