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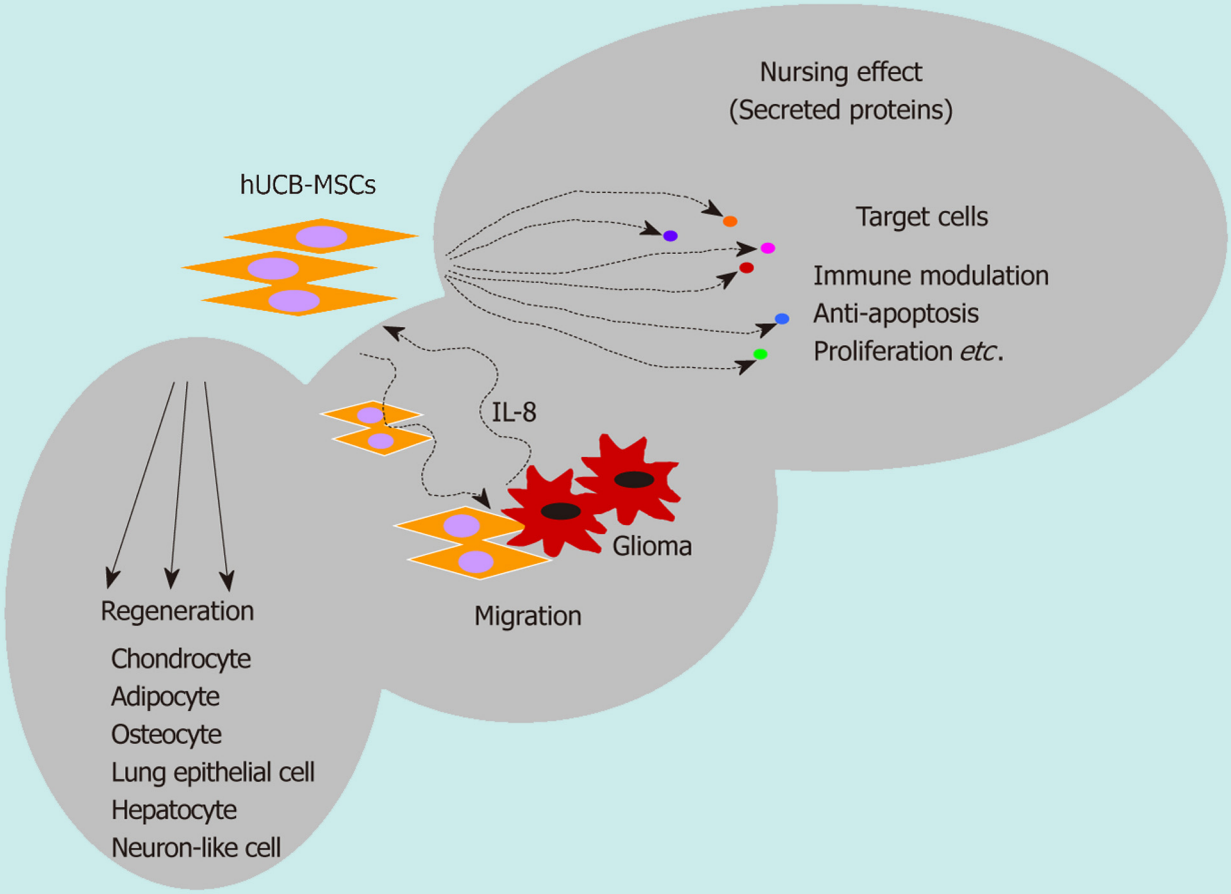

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EDITORIAL

Mesenchymal stem cells: From bench to bedside

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Abstract

Human mesenchymal stem cells (hMSCs) have tremendous promise for use in a variety of clinical applications. The ability of these cells to self-renew and differentiate into multiple tissues makes them an attractive cell source for a new generation of cell-based regenerative therapies. Encouraging results from clinical trials have also generated growing enthusiasm regarding MSC therapy and related treatment, but gaps remain in understanding MSC tissue repair mechanisms and in clinical strategies for efficient cell delivery and consistent therapeutic outcomes. For these reasons, discoveries from basic research and their implementation in clinical trials are essential to advance MSC therapy from the laboratory bench to the patient's bedside.

Key words: Mesenchymal stem cells; Cell therapy; Cell expansion and processing

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MESENCHYMAL STEM CELLS

Friedenstein and coworkers were the first to investigate the characteristics of the colony forming fibroblastic cells, which were isolated from the bone marrow by their selective adherence to tissue culture plastics[1]. Several other groups extended the pioneering work of Friedenstein *et al*[1] showing that these plastic adherent human cells derived from bone marrow were able to differentiate into a number of mesenchymal cell types including osteoblasts, chondrocytes and adipocytes[2-4]. These cells were called "mesenchymal stem cells (MSCs)" in reference to their high self-renewing properties and ability to form cartilage and bone, and were suggested to be responsible for the normal turnover and maintenance of adult mesenchymal tissues[5]. Although the initial application of MSC was to form feeder layers for hematopoietic stem cells, hence the alternate name "marrow stromal cells", MSC's therapeutic potential to cure a plethora of debilitating diseases was soon discovered and has generated significant excitement in the field of regenerative medicine[6]. Over the last two decades, the field of MSC has progressed rapidly from the preclinical to the early clinical trial arena for a wide range of diseases.

MSCs IN CELL THERAPY

MSCs hold tremendous promise for a variety of clinical applications. Ongoing clinical trials using human mesenchymal stem cell (hMSC) include ischemic stroke, multiple sclerosis, acute leukemia, graft-versus-host disease, critical limb ischemia, articular cartilage and bone defects among others (for the clinical trials presently tested, please see: www.clinicaltrial.gov). At the time of writing this article, there were about 90 clinical trials involving hMSC at various stages world-wide. The progress in clinical trials with MSCs in various diseases has been reviewed extensively[7-12].

Although the concept of cell-based therapy is not new and bone marrow transplants have been the standard of care for years, MSC-based cell therapies represent a

new generation of regenerative therapies that extend into other organ systems and meet pressing clinical needs for a broad range of diseases. MSCs are among the most widely used stem cell types in cell therapy owing to several favorable biological characteristics, including their convenient isolation from adult donors, ease of expansion in culture while maintaining genetic stability[13], lack of significant immunogenicity and feasibility for allogenic transplantation[14,15], and the homing capacity that facilitates intra-arterial/intravenous administration under minimally invasive conditions[16]. MSCs or MSC-like cells are now being isolated from blood[17], adipose tissue[18], trabecular bone[19], umbilical cord blood[20], and placenta[21] among other tissues. MSCs also have the remarkable property that they home to sites of tissue injury and institute repair, either by differentiating into tissue-specific cell phenotypes[22-25] or by creating a milieu that increases the capacity of the endogenous cells to repair tissue and modulates the immune response[26-28]. While the early studies have focused on cell differentiation, the recent results that demonstrate MSC's ability to repair tissues without significant engraftment or differentiation have led to new concepts for hMSC therapeutic effects. Critical features of this new paradigm are MSC's ability to not only secrete a rich mixture of soluble factors but also the ability to specifically respond to the immediate needs of the injured tissues. One specific example of the responsiveness of MSCs to microenvironment was the report that hMSCs injected into the hippocampus of mice following transient global ischemia decrease neuronal death by modulating inflammatory and immune responses. The transcriptomes of the hMSC changed with upregulation of 170 human genes that were largely involved in anti-inflammatory or anti-immune genes[29]. As another example, MSCs were activated by interferon-γ together with proinflammatory cytokines to express nitric oxide (NO) and several chemokines, suggesting that MSC-mediated immuno-suppression occurs through the concerted action of chemokines and NO[30]. MSC's responsiveness to the microenvironment of injured tissues suggests that the MSCs can be injected locally to enhance tissue repair, which could be one of the most useful cell therapy strategies.

While the original focus of hMSC's therapeutic potential was their ability to engraft and their plasticity, recent findings suggest that MSC's primary function is to inhibit immune responses and to establish a favorable microenvironment for tissue repair through immune modulation, down-regulation of inflammatory responses and paracrine effects[31]. Thus, the defining properties for hMSC should include not only their multi-lineage potential but also their robustness to respond to biological cues and to modulate the microenvironment. It is also likely that the therapeutic benefits of hMSC are a combined result of multiple contributing factors, generating both short-term tissue responses and long-term tissue repair and regeneration. For this reason, basic science studies are important to elucidate the controlling factors and to gain mechanistic insights underpinning MSC therapies.

CLINICAL APPLICATIONS OF MSCs

The beneficial outcomes from an increasing number of clinical trials using hMSCs without any major side effects has been a major driving force behind interest in MSCs' clinical application. As scientists learn more about MSC biology and tissue repair mechanisms, the encouraging clinical results, most notably in cardiac repair and bone disorders, have generated a growing enthusiasm.

Cardiac repair

A compelling clinical need exists in cardiovascular therapies to protect, restore and regenerate cardiomyocytes that are lost due to myocardial infarctions and heart failure. Bone marrow-derived cells, including both hematopoietic and MSCs, have shown remarkable clinical efficacy in terms of functional improvements including ejection fraction, ventricular volumes, infarct size and myocardial perfusion[32-34]. The functional improvement that occurred within 72 h was far earlier than would be expected for cell regeneration, leading to intense debate about repair mechanisms after cell transplantation[35]. The prevailing concept of stem cell efficacy has now shifted toward the cytokine-paracrine effects, which have been shown to modulate angiogenesis, inflammation, cytoprotection, metabolism and apoptosis. Despite the exciting possibilities that stem cell therapy have major beneficial effects on myocyte regeneration, inconsistent outcomes and, in some cases, poor engraftment and modest improvement have been reported in human trials[36-38]. These results highlight the need to understand the MSC tissue repair mechanisms and exact biology of stem cells in order to address the limitations such as the optimal cell type, mode of cell processing and delivery. The focus of improving and standardizing cell processing and delivery methods should be on enhancing cell engraftment while maintaining their therapeutic potency.

Bone disorders

MSCs have considerable potential for treatment of musculoskeletal disorders owing to their expansion capacity, immunosuppressive properties and ability to differentiate into bone and cartilage. Autologous bone marrow-derived MSCs have been used in fracture nonunion, osteogenesis imperfecta, and bone metabolic diseases, and demonstrated bone formation and limb function recovery in patients[39-42]. In addition, MSCs are also combined with scaffolds that are inductive or instructive to direct MSCs down specific lineage pathways and augment the therapeutic effect. Considerable *in vitro* and animal studies suggest MSCs have the potential for rapid bone regeneration and are the cell of choice in bone repair. However, in contrast with most studies in cardiovascular therapies, the numbers of patients studied in stem cell therapy for bone diseases and repair are relatively low and more long-term and sufficiently controlled clinical trials are needed to assess the therapeutic outcome. As MSCs are the progenitors responsible for the normal turnover of adult mesenchymal tissues and have

high responsiveness to tissue injury, "intelligent" materials that are able to recruit endogenous MSCs *in vivo* and direct them down specific pathways will be a useful therapeutic avenue.

PROMISE AND OBSTACLES OF MSC THERAPY

The last few years have witnessed a growing enthusiasm for the clinical application of MSC-based therapy. Despite the significant potential, challenges in MSC's clinical applications include low survival of transplanted cells, limited targeting capabilities, and low grafting efficiency and potency, which often requires use of a high number of cells to achieve therapeutic benefits. To date, clinical studies using stem cells have not been conclusive and are, in many cases, less impressive than what has been observed in preclinical models. A major obstacle limiting MSC clinical application is the lack of defining markers due to the inherent heterogeneity of MSC populations and variation associated with cell processing and expansion. The lack of standardization and variation in cell characterization and processing may help explain the discrepancies observed in some of the clinical studies[43]. Standardization is also critical for meaningful interpretation and comparison of experimental outcomes and understanding the mechanisms underlying the potential benefits of stem cells.

A hallmark of stem cells is their ability to expand in culture without phenotypic alternations. In the bone marrow obtained from human donors, hMSC's are rare and in the range of approximately 1 in 10^5 nucleated cells. Because of the low occurrence of MSC in bone marrow, only culture-expanded MSCs are likely to meet the demand in clinical application. However, DNA replication is not a perfect process and *in vitro* cell processing and expansion could induce potential changes to the cell and increase risks in their therapeutic applications. In addition to the safety concerns, the impact of culture expansion and cell processing on hMSC therapeutic potency is largely unknown and requires further investigation. Recent studies have shown that sequential passaging of MSC using standard culture methods has been associated with a decrease in expression of adhesion molecules, the loss of chemokine receptors, enlargement of cell size and lack of chemotactic response to chemokines, thus compromising their therapeutic potency[44-46].

Several recent studies have illustrated the increasingly recognized importance of cell processing of MSC for specific clinical indications. Le Blanc's group has recently shown that cryopreservation reduces the yield of *ex vivo* expanded MSC obtained from freshly harvested bone marrow mononuclear cells (MNC). In addition, MSC from fresh MNC were more potent in suppressing the lymphocyte responses in a mixed lymphocyte culture compared with MSC prepared from cryopreserved MNC[47]. In still another study, MSC pre-conditioned under hypoxic condition (0.5% O_2 for 24 h) increased expression of pro-survival and pro-angiogenic factors and enhanced the capacity of MSC to repair infarcted myocardium, owing to reduced cell death and apoptosis of transplanted cells, increased angiogenesis, and paracrine effects[48]. While these studies confirmed the seemingly obvious notion that MSC properties and functional capacity vary depending on the processing protocols, they also represent the beginning of an important research arena that addresses a bottleneck in MSC therapy.

PROSPECTIVE

Stem cells produce all multi-cellular tissues in the body in tightly controlled microenvironments. As a result, they are particularly sensitive to their immediate environmental cues. A case in point is the importance of a seemingly pedestrian factor of oxygenation for stem cell fate. Low oxygen tension, traditionally termed "hypoxia", is known to profoundly influence cellular events, cytokine physiology, and regenerative potential, and may in fact represent an "*in situ*" normoxia[49,50]. Although oxygen tension has been recognized as a developmentally important stimulus *in vivo*, it has not been adequately accounted for in *in vitro* cultures[51]. As the concept of MSC therapy shifted from the early proliferation-differentiation-engraftment assumption to the paracrine hypothesis, MSC therapeutic properties are now defined not only by their proliferative and multi-lineage potentials but also their ability to respond to and influence their immediate surrounding environments. To this end, basic and preclinical research will continue to play an important role in uncovering the dynamic interplay between stem cells and their microenvironments. Implementing these discoveries in clinical trials will be critical to advance MSC therapy from bench to a clinical reality.

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TOPIC HIGHLIGHT

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Hematopoietic stem cells in research and clinical applications: The "CD34 issue"

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Abstract

In this paper, experimental findings concerning the kinetics of hematopoietic reconstitution are compared to corresponding clinical data. Although not clearly apparent, the transplantation practice seems to confirm the basic proposals of experimental hematology concerning hematopoietic reconstitution resulting from successive waves of repopulation stemming from different subpopulations of progenitor and stem cells. One of the "first rate" parameters in clinical transplantations in hematology; i.e. the CD34+ positive cell dose, has been discussed with respect to the functional heterogeneity and variability of cell populations endowed by expression of CD34. This parameter is useful only if the relative proportion of stem and progenitor cells in the CD34+ cell population is more or less maintained in a series of patients or donors. This proportion could vary with respect to the source, pathology, treatment, processing procedure, the graft *ex vivo* treatment and so on. Therefore, a universal dose of CD34+ cells cannot be defined. In addition, to avoid further confusion, the CD34+ cells should not be named "stem cells" or "progenitor cells" since these denominations only concern functionally characterized cell entities.

Key words: Hematopoietic progenitors; Transplantation; Hematopoietic stem cells; Hematopoietic reconstitution; Granulocytopenia; CD34+; Functional stem cell definition; Immunophenotype

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EXPERIMENTAL HEMATOLOGY: SOURCES AND LESSONS

From the first experimental proof of the existence of hematopoietic stem cells provided by the classical experiment of Till and Mc Culloch[1] and from its consequences[2] (1961), a new discipline - experimental hematology - has developed. The first approach of experimental hematology is to characterize the functional heterogeneity of stem and progenitors cells by *in vivo* and *in vitro* functional assays; the second approach consists of searching for an immunophenotype characterizing each of the different subpopulations of stem and progenitors cells[3-6]. Although important advances have been made in terms of enrichment of stem cells by means of immuno-phenotypical properties, the initial functional characterization is still the only way

to prove the existence of the stem cell entities[7]. This functional definition could not be avoided; this can be illustrated by two major breakthroughs in stem cell biology: (1) induction of pluripotent stem cells from somatic cells[8-10]; and (2) initiating hematopoiesis from human embryonic stem cells[11].

Taken together, almost four decades of research on stem cells that exhibit a hematopoietic differentiation potential allowed an understanding of the functional heterogeneity of stem and progenitor cells, proposed a long time ago as the "generation-age hypothesis"[12]. This heterogeneity is the main factor leading to a very complex situation that does not allow simplification without losing some essential notions.

The first reports revealing this heterogeneity dealt with the phenomenon of hematopoietic reconstitution after engraftment. Two phenomena; i.e. the kinetics of red blood cell repopulation (erythrocyte repopulating ability-ERA) and the kinetics of granulocyte repopulation (granulocyte repopulating ability-GRA) were reported[13-15]. It was evident that these phenomena resulted from the activity of two distinct cell populations that are more immature than morphologically recognizable precursors of these two lineages, but less immature than the multi-lineage progenitors called "colony forming unit-spleen (CFU-S)" detected by the assay of Till and McCulloch[16-21]. The development of *in vitro* assays for clonogenic progenitors showed that these two repopulating activities result from two distinct populations of committed progenitors: those of granulocyte monocyte lineage (CFU-GM) and those of erythroid lineage (CFU-E, BFU-E)[21]. But these "repopulating activities;" i.e. "committed progenitors," are different from CFU-S[22], whose population is capable, if transplanted after lethal irradiation, to protect animals from acute radiation-induced lethality ("radio-protective ability")[23]. The CFU-S population has also been shown to be heterogenous; relatively less primitive CFU-S produced colonies 8 to 9 d after injection of hematopoietic cells and the other relatively more primitive CFU-S produced colonies 12 to 14 d after the injection. In fact these sub-populations of CFU-S are overlapping[24]. Furthermore, the "late" colonies growing 12 to 14 d from more primitive multipotential progenitors contain more primitive cells, which are responsible for short-term engrafted clone maintenance, known under the generic terms "pre-CFU-S" or "marrow repopulating ability-MRA"[25-27]. Actually, this is the first population that could be considered as a real stem cell population according to current standards. Even more primitive stem cells have subsequently been found, allowing long-term maintenance of hematopoiesis after engraftment[28].

The previous paragraphs summarize 25 years of work, which enabled realization that hematopoietic stem cells and progenitors are organized as a continuum of descendant cell populations having a decreasing proliferative capacity and decreasing self renewal ability, starting from the most primitive stem cells to the last progenitors preceding precursors. In animal experimental models, the reconstitution of hematopoiesis after engraftment and consequent repopulation of peripheral blood results from successive waves of repopulation. This phenomenon stems from the heterogeneity of stem and progenitor cells since less primitive cells take less time to develop morphologically recognizable hematopoietic cells and *vice versa* for more primitive progenitors and stem cells. Some results suggest, however, that long term reconstitution could stem from short term reconstituting stem cells that are activated and exhausted in a successive manner[29-30]. This question does not interfere with the phenomenon of initial reconstitution after transplantation, for which the mechanism is well established and accepted. In summary, the works of experimental hematology imply that for a rapid and long term hematopoietic repopulation, a sufficient number of both stem cells and committed progenitors (of all categories) should be injected. With the development of *in vitro* cultures for the detection of human committed progenitors, as well as *in vivo* xenogenic transplantation models for the detection of human stem cells, the main points initially established in animal models have been confirmed for hematopoietic stem and progenitors cells issued from three main "human" sources: bone marrow, peripheral blood after mobilization and placental (cord) blood[5,31].

The concept of *ex vivo* expansion is derived directly from this knowledge. It is based on a very attractive idea to increase the number of cells and progenitors (aimed to accelerate hematopoietic reconstitution) in order to insure a secure and favorable long-term outcome of transplantation. As a matter of fact, for clinicians, the first objective of an *ex vivo* expansion is shortening the period of post transplantation agranulocytosis. The duration of this period varies between 1 and 4 wk depending on the source of transplanting cells [peripheral blood after mobilization, bone marrow, and placental (cord) blood].

On the basis of experimental hematology data from animal models[13-22], duration of this period depends mostly on the number of relatively mature progenitors present in populations of transplanted cells. On the other hand, experimental data demonstrate that, for long-term reconstitution, the presence of more primitive stem cells is required[25-28]. Accordingly, the ideal *ex vivo* expansion should allow amplification of both committed progenitors and stem cells.

IMPLEMENTATION IN CLINICAL HEMATOLOGY

From this experimental work, clinical hematology adopted the principle of hematopoietic stem cell transplantation. This practice started with bone marrow cells but other sources were preferred subsequently: hematopoietic progenitors and stem cells mobilized to peripheral blood as well as those from placental (cord) blood. The first bone marrow transplantations were allogenic, aimed to reconstitute the hematopoietic system of humans irradiated in a nuclear accident[32]. Since then, hematopoietic cell transplantation

as a clinical discipline yielded a tremendous amount of knowledge, not only related to stem cell biology, but also for immunology (e.g. discovery of the HLA system). In spite of this fact, the development of clinical transplantation sometimes neglects some fundamental points of experimental hematology. We discuss one of these points in this review.

Total CD34+ cell dose issue

Since the beginning of clinical transplantation practice, the total number of viable cells has been considered as a main parameter in transplantation. Though polymorphonuclear cells, monocytes, and lymphocytes do not provide hematopoietic reconstitution after transplantation, the total cell number is still considered as a first rate qualifying and prognostic factor in transplantation, especially for placental (cord) blood cells. Indeed, in most papers describing transplantation of cells issued from hematopoietic sources in the steady state, there is a correlation between engraftment, kinetics and the total cell number in the graft[33]. This correlation results from the fact that the concentration of stem and progenitor cells in hematopoietic tissues in the steady state, or after some standard therapeutic protocols, is more or less constant. Thus, the increase in total cell numbers also means an increase in stem and progenitor cell numbers. In addition, this parameter is easily and rapidly determined. Taken together, its usefulness has been confirmed. Of course, it would be wrong to consider that, due to this correlation between total cell number and transplantation outcome, the engraftment is achieved by total cells instead of stem cells.

The possibility of detecting CD34+ cells enabled researchers and technologists to approach a non-differentiated cell population containing most hematopoietic progenitors and stem cells[34], but this was not specific. Vascular endothelial cells, perivascular dendritic cells, hair follicle "stem" cells, spindle shaped cells of eccrine glands cells, for example, also express CD34[35]. This molecule, (also known as, e.g. "podocalyxin-like protein", "thrombo mucin", "gp135", *etc.*) belongs to a family of proteins ("CD34-family") that have overlapping expression patterns[36]. CD34-family proteins (CD34, podocalyxin, and endoglycan) have a serine-, threonine-, and proline rich extracellular domain that is extensively O-glycosylated and sialylated (90-170 kDa). The function of CD34 family members has not yet been definitively elucidated. However, several roles have been ascribed to these proteins; for example, the proliferation-promoting effect, differentiation-blocking effect on progenitor cells, enhancement of trafficking and migration of hematopoietic cells, and a role in cell morphogenesis[36]. Despite this expression pattern, nonspecific to hematopoietic tissue, and an elusive physiological role, the CD34 protein has become, in the minds of many in the biomedical community, the main marker endowing hematopoietic stem and progenitor cells. Furthermore, most clinicians and biologists who are not directly involved in stem cell research have a tendency to add the term "stem cells" each time they say or write "CD34+". This tendency has been a permanent source of misunderstanding and confusion and it heavily affects experimental and clinical hematology. It should therefore be repeatedly stressed that the fact that the majority of hematopoietic stem and progenitors cells express CD34+ does not mean that all CD34+ cells are stem cells or progenitors. The CD34+ cell population is very heterogeneous[34]. For example, in the CD34+ population of placental (cord) blood, 30% to 50% are progenitors (CFU-GM, BFU-E, CFU mix, and CFU-Mk) and only a small percentage are primitive stem cells. Approximately one half of the CD34+ cell population does not exhibit either progenitor or stem cell functional properties. Some stem cells do not express CD34+ in a steady state[37] and expression of this molecule could be reversible and not related to functional capacities of stem cells[38]. Here again, the CD34+ cell count in different cell populations derived from hematopoietic tissues in a steady state or mobilized in peripheral blood has been confirmed as a useful parameter of the graft concerning the kinetics of engraftment[39-44], although only a small fraction of these cells have stem cell characteristics. The dose of CD34+ cells correlates well with the dynamics of hematopoietic reconstitution compared to total cell number. This results from the fact that the proportion of progenitors in stem cells inducing "transitory" engraftment in the CD34+ population is higher than in other subpopulations. It is also relatively stable for the tissue in question. In addition, it is easy to get the count of CD34+ cells by immuno-staining and flow-cytometry. Thus, the number of CD34+ cells became a main parameter of graft quality control. Since rapidity of hematopoietic reconstitution correlates with the number of CD34+ cells per kilo of patient weight, this approximation induced a "mental shortcut" in clinical hematology; the term "CD34+ cells" is frequently equated with the term "stem cells". On the contrary, experimental hematology considers the term "stem cell" as a functional entity (or state)[7]. Even a very complex and sophisticated procedure aimed to isolate "stem cells", based on several immuno-phenotypic markers and combined with metabolic properties, only enabled a high degree of enrichment and not a completely pure stem cell population[45]. For example, Lin- CD34+ CD38- fraction from placental (cord) blood only contains 1%-2% of stem cells detectable by a functional *ex vivo* assay[46]. In addition, if steady state is disrupted, as it is in *ex vivo* expansion cultures, for instance, the relationship phenotype/function is less evident or even non-existent[47-51].

Clinical vs experimental

In general, it is more difficult to follow the specific effect of one variable in clinical rather than experimental situations. The individual variations of cellular parameters in humans are larger than in rodents. The treatment of humans should be effected within the requirements of clinical trials. In addition, the preparation of the graft is restrained to only accepted and validated procedures. After all, the interference of different human pathologies,

as well as previous treatments and therapeutic approaches, could have a big impact on the effects of transplantation. These are only some of the reasons why it is sometimes difficult to reproduce the same effect on humans that was demonstrated in animal experimentation. The apparent absence of correlations in some clinical trials, however, between two variables that correlated in animal trials, does not mean that the principle is automatically erroneous.

This should be considered in the issue of hematopoietic reconstitution after transplantation. Many papers have been published demonstrating a positive correlation between the total number of cells and the number of CD34+ cells and hematopoietic reconstitution. Determination of hematopoietic progenitors on the basis of their colony-forming capacity in culture is less practical and more time consuming than determining CD34 expression; therefore, the number of hematopoietic progenitors has not been systematically taken into consideration in analysis of hematopoietic reconstitution[33,39-44]. However, in some reports, these parameters were properly analyzed. These analyses almost always showed that the best correlation is between committed progenitors and rapidity of hematopoietic reconstitution[52-54] in comparison with total cells and CD34+ cells. Other studies have shown the absence of correlation between the total cell number, CD34+ number and clinical and hematologic outcomes[55]. This confirms a relative progenitor and stem cell source-dependent value of these parameters [unfortunately, the progenitor (CFC) number analysis was not shown]. Furthermore, short term repopulating cells, previously demonstrated in animal models, also exists in human grafts. In bone marrow grafts, for example, these short term repopulating cells have clearly demonstrated a hematopoietic reconstitution inferior to 100 d[56]. These stem cells, found in sub-populations CD34+ and CD34- and CD34+ HLA-DR-, are not correlated with a long term hematopoietic reconstitution (between 100 d and a year post transplantation). This late reconstitution, however, is correlated with CD34+ cell number, due to the presence of very primitive stem cells inside this heterogeneous population, as mentioned above[56].

These discoveries confirm that human stem cell biology is not an exception with respect to other vertebrates. This information is in favour of the "expansion concept," which postulates that *ex vivo* amplification of committed progenitors should accelerate hematopoietic reconstitution after transplantation. We could not analyze here all clinical trials that were recently reviewed dealing with the transplantation of bone marrow, peripheral blood, and cord blood hematopoietic cells after *ex vivo* expansion[57]. The initial inefficiency of this approach, however, was due to inefficient *ex vivo* protocols and/or to the study design rather than an erroneous concept. Some, however, demonstrated a positive effect on hematopoietic reconstitution after transplantation, decreasing the incidence of neutropenic fever, reduction of red blood cell transfusions, and the diminution of the duration of hospitalization[58,59]. A trial carried out with a combination of cytokines, showing a high pro-differentiation power, enabled a relatively modest expansion of total cells and progenitors. Although this trial did not provide an acceleration of hematopoietic reconstitution, it is important because transplanted cells failed to maintain short and long term reconstitution after aplasia[60]. With current knowledge, it could be proposed that the stem cells with short term and long term repopulating capacities have been exhausted in expansion cultures due to the culture conditions, especially to IL-3 and IL-1 association and the exposure of the culture to ambient oxygenation. Thus, this trial underlines the importance of the presence of primitive stem cells in a graft. Furthermore, it firmly demonstrates that the number of CD34+ cells only is not a universally appropriate parameter of the graft quality, since the primitive stem cells could be absent. Also, if a graft, as in this case an expansion product, is composed exclusively of committed progenitors without stem cells, it could only ensure a transient engraftment.

The first really successful expansion protocol[61,62] confirmed that hematopoietic reconstitution depends on the functional sub-populations of progenitor and stem cells that should be present in a graft. In addition, it presents a very interesting example of the phenomenon called "dissociation phenotype-function". During the pre-clinical development of this expansion procedure, as well as in expansion for clinical trials, we found that the expansion of progenitors with a mean value of 27 fold was accompanied with an expansion of CD34+ cells of only 3.5 fold[62,63]. In terms of absolute number, we get almost twice the number of committed progenitors than CD34 cells. This means that, in the course of *ex vivo* expansion, the culture generated the progenitors that do not express CD34 antigen (see the studies related to the transient expression of CD34)[38]. Thus, the predictable value of the CD34+ cell count could be questioned for expansion products. Indeed, the results derived from clinical trials point to the absence of correlation between the number of CD34+ cells in a graft and the duration of post-transplantation agranulocytosis[62]. On the contrary, the number of committed progenitors was well correlated with the acceleration of post transplantation hematopoietic reconstitution[62].

Concluding remarks

On the basis of experimental data, the capacity of a CD34+ cell population to reconstitute hematopoiesis quickly after engraftment, as well as in the short- and long-term perspective, depends on the presence (in sufficient number) and proportion of functionally very different CD34+ sub-populations. This proportion should vary with respect to the source (e.g. bone marrow peripheral blood[64], cord blood[5,31]), pathology, treatment, processing procedure, the graft *ex vivo* treatment[62] and so on.

For all these reasons, the same number of CD34+ cells could give completely different results related to the rapidity of hematopoietic reconstitution and the short and long term maintenance of hematopoiesis. Considering this, it would not be expected that the number of CD34+

cells would become the universal "first rate" parameter for clinical transplantation, and that a universal CD34+ cell dose could be defined.

Also, to avoid further confusion in research and clinical practice, the heterogenous population of cells endowed by CD34+ antigen expression should not be named as "stem cells" or "progenitor cells". These denominations only concern functionally characterized cell entities.

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TOPIC HIGHLIGHT

Philippe Bourin, MD, PhD, *Series Editor*

Human pluripotent stem cells: From biology to cell therapy

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Abstract

Human pluripotent stem cells (PSCs), encompassing embryonic stem cells and induced pluripotent stem cells, proliferate extensively and differentiate into virtually any desired cell type. PSCs endow regenerative medicine with an unlimited source of replacement cells suitable for human therapy. Several hurdles must be carefully addressed in PSC research before these theoretical possibilities are translated into clinical applications. These obstacles are: (1) cell proliferation; (2) cell differentiation; (3) genetic integrity; (4) allogenicity; and (5) ethical issues. We discuss these issues and underline the fact that the answers to these questions lie in a better understanding of the biology of PSCs. To contribute to this aim, we have developed a free online expression atlas, Amazonia!, that displays for each human gene a virtual northern blot for PSC samples and adult tissues (http://www.amazonia.transcriptome.eu).

Key words: Pluripotent stem cells; Embryonic stem cells; Therapeutics; Cell reprogramming; Cell proliferation; Cell differentiation

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INTRODUCTION

Pluripotency is the ability of a cell to differentiate into any cell type of the developing or adult animal or human. Stem cells that are pluripotent, while not being malignant, were first discovered in mice in 1981[1], sparking radical new research avenues such as *in vitro* studying of early embryo development, cell differentiation and genetically modified animals. For this latter application of pluripotent stem cells (PSCs), Martin Evans earned the Nobel Prize for Medicine in 2007[2]. Seventeen years later, James Thomson and colleagues succeeded in deriving human PSCs from human embryos issued from *in vitro* fertilization, generating

human embryonic stem cells (hESCs)[3]. This feat provided a completely new source of cells for biomedical applications[4,5]. Recently, the field of pluripotency was again shaken by the breakthrough discovery of Kazutoshi Takahashi and Shinya Yamanaka, evidencing that a differentiated somatic cell was amenable to complete dedifferentiation into PSCs by the over-expression of only four transcription factors (TFs)[6,7]. This technique of generating induced pluripotent stem cells (iPSCs) has provided an unrivaled means to understand the production and maintenance of pluripotency, resolved the ethical issues of the destruction of human embryos connected to hESCs, and outlined a method to use PSCs in medicine in an autologous setting that is more practical than therapeutic cloning. We will review here the specific determinants of pluripotency, the requirement for PSC culture, the expected use of PSCs in cellular therapy, and the pitfalls that must be anticipated and avoided to bring PSCs safely to therapeutics.

INTRINSIC MOLECULAR DETERMINANTS OF PLURIPOTENCY

Gradually, the molecular mechanisms that underlie pluripotency are becoming unveiled. The determinants of pluripotency can be divided into two broad categories: intrinsic determinants; i.e. cell-autonomous factors, for example, TFs, and extrinsic determinants that are non-cell autonomous, for example, growth factors[8]. Strikingly, intrinsic determinants are largely shared between mouse and human PSCs, whereas extrinsic determinants are often radically different between these two species. This last point accounts for, at least in part, the extended period that elapsed between the identification of ESCs in mice and in humans. The core transcriptional circuitry, the major determinants of intrinsic pluripotency, is composed of the TFs *OCT4*, *NANOG* and *SOX2*[9]. These three TFs repeatedly co-occupy the promoters of their target genes, including themselves, thus inducing a positive regulatory loop of pluripotency. Paradoxically, the core pluripotency TFs not only occupy the promoters of genes involved in pluripotency, putting them in close association with RNA polymerase Ⅱ, but also promoters of genes that are inactive in PSCs and linked to cell differentiation, such as *PAX6*, *HAND1* or *ISL1*, by placing them in proximity to proteins of the polycomb group[10].

The fact that for differentiation genes the cognate promoters are simultaneously co-occupied by the core pluripotency TFs and the polycomb repressive complex 2 subunit SUZ12, leading to a repressive chromatin modification by trimethylation at histone H3 K27 (H3-K27me3), indicates a link between pluripotency and the epigenome. Several lines of evidence suggest that PSCs are characterized by a very specific chromatin state[11]. Global gene expression analyses by whole-genome tiling arrays have shown widespread transcription in coding and non coding regions in ESCs, as opposed to differentiated cells in which the transcriptional landscape subsides as differentiation proceeds[12]. This distinct expression profile in PSCs is associated with a high expression of chromatin remodeling genes, such as *TOP2A*, *DNMT3B*, *JARID2*, *SMARCA5*, *CBX1* or *CBX5*[13]. While a majority of promoters are occupied by nucleosomes with H3K4me3 modifications, typically associated with an open chromatin structure and active transcription, not all H3K4me3-modified promoters are transcriptionally active[14]. One explanation for this contradiction is the concomitant repressive modification by H3K27me3, hence forming "bivalent" modifications[15,16]. The bivalent H3K4me3/H3K27me3 modification can easily switch to a monovalent modification, chiefly H3K4me3, and therefore the bivalent mark was proposed to be an indicator of genes specially poised to initiate transcription during differentiation. Bivalent modifications were first found in ESCs, but were subsequently also found in fully differentiated cells, suggesting a mechanism that is general and not restricted to ESCs. Another explanation can be found in the recent findings that the most cell-type-specific histone modification pattern is observed at enhancers and not at promoters[17]. The mechanisms that are necessary to keep this chromatin state may involve the chromatin remodeling factor *Chd1*, since its ablation disrupts PSC differentiation capacities[18]. Hence, the global picture that emerges is that ESCs have an open chromatin largely devoid of heterochromatin, priming their genes for transcription at later stages of development, thereby accelerating the full transcription activation required by cell differentiation.

Niall Dillon's group has reported that genes that are transcriptionally silent in ESCs are nonetheless subject to preinitiation complex assembly but are simultaneously targeted by the proteasome[19,20]. Their data suggested that the 26S proteasome promotes a dynamic turnover of TFs and Pol Ⅱ, binding at tissue-specific gene domains in ESCs, which would restrict permissive transcriptional activity but keep the genes in a primed state for later activation. In line with the potential role of the proteasome machinery in the distinct transcription regulation of PSCs, we recently reported the overexpression of several genes involved in the canonical ubiquitin-proteasome pathway[13]. Significantly overexpressed in hESCs were genes coding for enzymes from the three (E1/E2/E3) ubiquitination classes; i.e. the E1 ubiquitin-activating enzyme *UBE1C*, the E2 ubiquitin-conjugating enzymes *UBE2G1*, *UBE2V1* and *UBE2V2*, and the E3 ubiquitin protein ligases *UBE3B* and breast cancer 1, early onset (*BRCA1*), as well as four catalytic β proteasome subunits (*PSMA2*, *PSMA3*, *PSMA4* and *PSMA5*), three regulatory subunits, the ATPase *PSMC6*, and the non- ATPase *PSMD10* and *PSMD11* from the proteasome machinery. This peculiar expression of the proteasome in PSCs was correlated with an acute sensitivity of hESCs to proteasome inhibitors.

In addition, other genes are also overexpressed in PSCs, including numerous zinc finger TFs that could play a role in the intrinsic determination of the pluripotency state. We have re-analyzed a large panel of hESC transcriptome studies and have established a common list of genes invo-

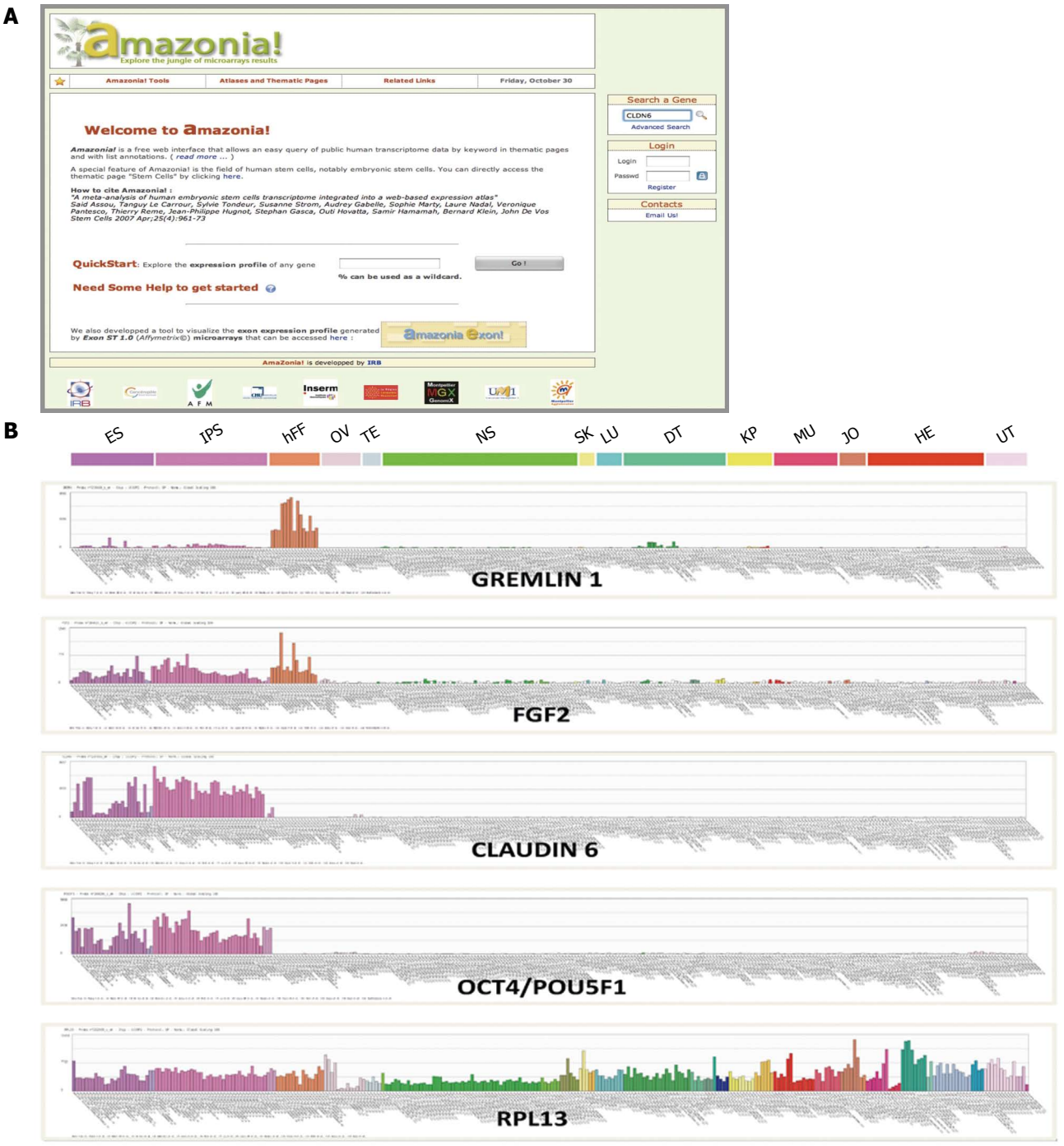

Figure 1 Visualization of gene expression in PSCs and comparison with somatic cells. A: The *Amazonia!* web Atlas interface (http://www.amazonia.transcriptome.eu); B: Expression bar plots, generated with *Amazonia!*, for RPL13, a ubiquitously expressed gene, OCT4/POU5F1 and CLAUDIN 6 as highly PSC-specific genes, FGF2, a major human PSC growth factor expressed as an autocrine loop and by human fibroblast cells, and GREMLIN 1, an inhibitor of BMPs secreted by human fibroblast feeder cells. ES: Human embryonic stem cells; iPS cells: Induced pluripotent stem cells; hFF: Human foreskin fibroblasts; OV: Ovary & oocytes samples; TE: Testis; NS : Nervous system; SK: Skin; LU: Normal lung; DT: Digestive tract; KP: Kidney & prostate; HM: Heart & muscle; JO: Joint; HE: Normal hematological samples; UT: Uterus; PSCs: Pluripotent stem cells. Y-axis is the microarray signal value, obtained by MAS5 normalization with a TGT at 100 using Expression Console (Affymetrix, Santa-Clara, CA).

lved in pluripotency[21]. Importantly, we have made the transcriptome of PSCs available through *Amazonia!*, a web-based atlas of human gene expression that compiles a selection of publicly available transcriptome datasets and is freely accessible through a user friendly interface to the research community. Using this interface, one can easily grasp the very specific expression pattern of the core pluripotency TFs in PSCs, as well as in the central nervous system, upper digestive, airway tract, *etc*. (Figure 1).

Another level of cell fate regulation that takes place in PSCs is micro RNA (miRNA). Certain miRNAs have a high expression in hESCs and are lost upon differentiation into embryoid bodies, such as the miR-302 and miR-371 clusters[22,23]. Conversely, miR-145 expression increases

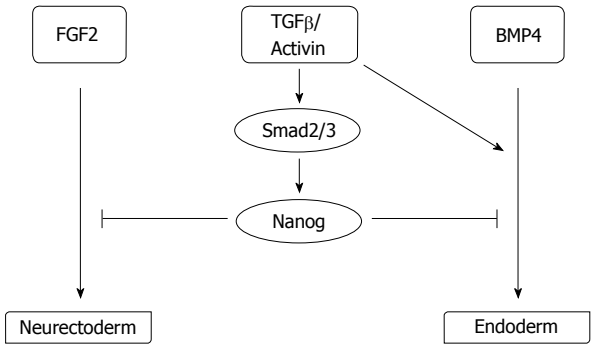

Figure 2 Model explaining the role of the major human PSC growth factors identified to date, FGF2 and TGFβ/Activin, based on Vallier *et al*[36] and Xu *et al*[43].

during PSC differentiation and directly represses *OCT4*, *SOX2*, and *KLF4*, thus blocking pluripotency by a negative feedback loop[24]. In addition, the pluripotency gene *LIN28* was observed to hinder the biogenesis of some miRNAs, such as the processing of pri-let-7 miRNAs[25]. These findings explain the complete absence of mature miR-let-7 in ESCs.

EXTRINSIC MOLECULAR DETERMINANTS OF PLURIPOTENCY

While intrinsic pluripotency determinants ensure that pluripotency is maintained, extracellular signals alter this undifferentiated state and drive the PSCs to differentiation. Hence, pluripotency is under tight control by extrinsic determinants; i.e. growth factors and other soluble factors, cell-to-cell contact, and the extracellular matrix and O_2 level. As mentioned above, growth factor requirements vary widely between mice and humans. For maintenance of pluripotency, mice ESCs rely on leukemia inhibitory factor (LIF), *via* a signaling cascade involving the phosphorylation of STAT3[26], and on bone morphogenic proteins (BMPs), *via* the expression of Id proteins[27]. By contrast, hESCs are indifferent to the action of LIF[28-30], and are highly sensitive to the action of BMPs, which induce hESC differentiation[31]. Human pluripotency is favored by the action of FGF2[32]; in contrast, an autocrine FGF loop in mouse ESCs drives their differentiation unless the action of this loop is counterbalanced by LIF[33]. The debate remains open as to whether the differences between growth factor requirements in mice and humans are secondary to speciation or rather to a different origin of developmental stage as suggested by the identification of epiblast stem cells in mice whose growth is dependent upon activins and FGF2[34]. Other growth factors are important for human pluripotency, such as TGFβ and activins[35,36], neurotrophins[37], GABA[38], sphingosine-1-phosphate[39], WNTs[40], IGFs[41], and EGF family members such as Heregulin[41] or pleiotrophin[42]. The role of the TGFβ/Activin pathway is essential as it induces the expression of NANOG *via* SMAD2/3, which in turn counteracts the induction of neurectoderm by FGF2 or endoderm by BMP4 signaling[36,43] (Figure 2). Noggin, a BMP inhibitor, has also been described as promoting pluripotency in combination with a high concentration of FGF2[32].

The role of cell-to-cell contact (stem cell-stem cell or stem cell-feeder) is clearly demonstrated by the well-known difficulty to clone PSCs, due to a high apoptosis rate after enzymatic dissociation. This dependency can be reversed, at least partially, by the selective inhibition of the rho-associated kinase using the pharmacologic compound Y-27632[44,45]. Similarly, PSCs are tightly dependent on their attachment to a feeder layer or a synthetic matrix. This dependency could be due to anoikis, a subtype of apoptosis provoked by detachment of adherent cells from their matrix[46].

PSC cultured on plastic undergo rapid differentiation and apoptosis, exemplifying the need for these cells to be on an extracellular matrix. Historically, a feeder of irradiated or mitomycin-C treated murine embryonic fibroblasts (MEFs) was used to derive and maintain in culture the first ESC lines[1,3,47]. This technique is still widely used because of its low cost and high efficiency in PSC maintenance. MEFs can be replaced by human fibroblasts such as foreskin fibroblasts[48]. These feeder cells produce soluble factors, such as the BMP inhibitor GREMLIN 1[49], or the pluripotency promoting growth factor FGF2 (Figure 1B), but also numerous extracellular matrix components. In line with this observation, *in vitro* culture protocols have been developed that replace feeder cells by various purified or unpurified matrices such as laminin[50], collagen Ⅳ/fibronectin/laminin/vitronectin[38], vitronectin[51] or Matrigel, which is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, rich in extracellular matrix proteins[50]. It should be noted that, in many of these matrix conditions, hESC maintenance requires the use of either MEF- or foreskin fibroblast-conditioned medium or defined medium such as TeSR1 (see below), suggesting that some soluble proteins secreted by the feeder cells are necessary to compensate for the still incomplete synthetic matrices that have been tested.

Another important factor in hESC maintenance is the O_2 level. Several papers have reported the role of low O_2 (3%-5%) tension in preventing hESC differentiation[38,52,53]. These O_2 conditions are similar to those required for early human embryo development. However, it should be conceded that most *in vitro* culture protocols maintain their hESCs under high O_2 (20%) tension due to obvious technical and cost constraints.

DRIVING PSCs TOWARD CELLS OF MEDICAL VALUE: TUNING DIFFERENTIATION

As hESCs can differentiate into virtually any cell type, they could theoretically cure any illness resulting from the loss of functional cells. But one crucial issue is to determine which hESC-derived cell population will be

most helpful. It is now clear that undifferentiated PSCs should not be used for cell repair, as PSCs are highly proliferating cells that can, upon injection, form non malignant tumors of undifferentiated and differentiated cells that should be called teratomas, as they are formed by the association of "somatic tissue and their immature (fetal) precursors derived from more than one of the three embryonic germ layers"[54]. The occurrence of teratomas is not systematic. But the risk of teratoma development is obviously not acceptable in any clinical application. For instance, the transplanting of low doses of undifferentiated murine ESCs (1000-2000 cells) into the striatum of a rat model of Parkinson disease resulted in a well oriented dopamine neuron differentiation and was associated with a clinical improvement in 14 of 19 animals that had been successfully grafted, but resulted in the growth of an undifferentiated cell population and the death of 5 of the 19 animals[55]. In line with these observations, Roy *et al*[56] treated a similar rodent model with hESCs differentiated into a cell population highly enriched in dopamine neurons obtained by successive culture steps, including the co-culture with fetal human astrocytes, and obtained significant improvement of the treated animals as compared to the sham-treated. While an important human dopamine neuron population was observed at the periphery of the injection site, in close contact with the rat glia, the center of the injection site was filled with immature nestin-positive and proliferating human neural precursors. These observations suggested that the cell preparation contained the appropriate dopamine neuron population, but still contained poorly differentiated and proliferating cells, whose developmental and tumor potential is not well known. This experiment gave reason for a word of caution against the injection of unpurified ESCs, even after *in vitro* differentiation. A similar observation was recently reported by Aubry *et al*[57] These authors injected into the quinolinate lesioned right striatum of immunocompetent rats a population of hESCs differentiated into DARPP32-expressing striatal neurons. After 2 mo, the animals manifested lethargy, weight loss and hemiparesis, caused by a massive outgrowth of the human neural progenitor injected into the striatum. The answer to this paramount problem raised by PSCs could be cell sorting after differentiation. Darabi *et al*[58] set up a protocol to regenerate muscle in dystrophic *mdx* mice using murine ESCs. The investigators determined that expression of PDGFβ-R, a marker of paraxial mesoderm, and absence of Flk-1, a marker of lateral plate mesoderm, identified a cell population of myogenic progenitors that could be purified by flow cytometry cell sorting. Without cell purification, the animals developed teratomas, formed by cells originating from the donor, containg keratinocytes and cartilage formation, at the injection site; but after cell purification, this major side effect was eliminated, strongly supporting the idea that undesired cells, including undifferentiated cells, must be eliminated by cell sorting before *in vivo* transplantation.

While there seems to be a consensus to exclude undifferentiated cells, the level of differentiation to be achieved for clinical use of PSCs is still an open question. Naturally, the answer to this question will be tissue-dependent, or maybe even disease-specific. To generate cardiomyocytes, some authors have proposed a very brief time of *in vitro* differentiation, as short as 48 h, reducing the differentiation step to a simple cardiac-commitment step using BMP2[59]. By contrast, most neuron differentiation protocols are multi-step, several weeks long protocols[56,57]. Hematopoietic differentiation also requires long and complex culture steps, usually including the overexpression of the HOXB4 TF[60,61]. Numerous pre-clinical trial studies have convincingly showed that PSCs can be differentiated into cells with the capacity for tissue repair, but there is still a long way to go before all the differentiation issues are solved[59,62-64]. A recent benchmark comparison of different sources of human hepatocytes transplanted into Alb-uPAtg$^{(+/-)}$Rag2$^{(-/-)}$Gama c$^{(-/-)}$ mice suggested that primary adult hepatocytes were the best source of cells for attaining a significant liver repopulation, while fetal hepatocytes ranked second best and hepatocytes derived from ESCs worked poorly[65]. Though a huge amount of work is still needed to improve our hepatic differentiating protocols for PSCs, this study clearly highlights the fact that current protocols mainly generate hepatocytes with a fetal phenotype; i.e. low expression of homeostasis and detoxification genes, persistence of β-feto protein, that are not best suited for liver regeneration[66,67]. Overall, the *in vitro* transformation of PSCs into cell drugs is still in its infancy stage and further work and testing in pre-clinical studies is needed to improve these protocols.

GOOD MANUFACTURING PRACTICE IN CLINICAL-GRADE CELL GENERATION AND DIFFERENTIATION

The use of differentiated PSCs suitable for human therapy will require the same rigorous manufacturing as for any cell therapy product. As pointed out above, many differentiation protocols are based on extensive manipulation, involving many successive reagents, co-culture steps and several weeks of incubation at 37 ℃. Any constituent that will come in contact with the PSCs will have to meet the safety requirements of regulatory bodies. Several academic teams have already published defined or xeno-free media that could be used to develop clinical grade PSCs. The use for non-human materials bears a risk of transmitting pathogens. The elimination of animal serum is also an important step because hESCs cultured with animal products or animal cells express Neu5Gc, a nonhuman sialic acid that could be immunogenic if these cells were to be used for cellular therapy[68]. Henrik Semb's group has described a protocol using 20% of human serum instead of fetal calf serum or knock-out serum replacement (KO-SR), and have derived a new hESC line in these conditions[69].

Ludwig *et al*[38] have proposed a fully defined medium for PSC culture, based on the analysis of the expression of cell-surface receptors of hESCs and the finding that some of the ligands of these receptors have a positive effect on pluripotency (FGF2, ClLi, GABA, pipecolic acid, and TGFβ). This medium was termed TeSR1 and, in combination with human laminin, collagen Ⅳ, fibronectin and vitronectin, was able to sustain the derivation of two new hESC lines, demonstrating that the maintenance of pluripotency was not restricted to culture-adapted subclones of hESCs[38]. A modification of this medium, consisting of the replacement of human albumin with its bovine counter part, the replacement of human FGF2 with zebrafish FGF2, and the use of Matrigel instead of the purified human matrices, has been commercialized and is now widely used as mTeSR1 world wide[70]. Other xeno free media have been documented, but all compare poorly with fibroblast feeder/KO-SR standard culture conditions when tested on hESCs that were mechanically derived and passaged[71].

Another issue is the feeder cell layer that imparts complexity in cell handling and a risk of pathogen transmission, either for human or murine feeder cells. Therefore, both on scientific and medical grounds, substitution of the co-culture system by a synthetic matrix would be preferable. As early as 2001, the team of Melissa Carpenter proposed Matrigel (see above) or laminin as a replacement for MEFs, but only if using MEF-conditioned medium[50]. Numerous other proposals have been made since, such as the use of MEF sodium deoxycholate extract, which does not solve the xenogeneic source of the matrix but resolves the practical conundrum of the co-culture system[72], human fibroblast extracts[73], a mix of human purified extracellular components[38], and recombinant vitronectin on its own[51].

A recent twist in the domain of xeno-free PSC culture was the ability of such culture conditions to generate iPSCs. Several recent publications have illustrated this technical possibility, such as the use of TeSR1 and Matrigel[74] or a human plasma-derived cell culture additive called F44, obtained through cold-ethanol industrial plasma fractionation[75].

MAJOR HURDLES TO OVERCOME

The use of PSCs for clinical applications raises several issues that must be carefully addressed. These difficulties are: (1) cell proliferation; (2) cell differentiation; (3) genetic integrity; (4) allogenicity; and (5) ethical issues.

As noted above, PSCs are characterized by an abbreviated G1 phase of the cell cycle, resulting in sustained cell proliferation. Therefore, the injection of undifferentiated PSCs carries the risk of inducing teratomas, which consist of the non-malignant proliferation of PSCs associated with multilineage and uncontrolled cell differentiation, both of which are unwanted and deleterious[54]. An open question is the extent of differentiation necessary to prevent any risk of teratomas at the site of injection and the relevant cell markers that can be used to sort the cells. Obviously, the response to this answer will be cell type-specific, but one can anticipate that the loss of one or several (signature) pluripotency markers may turn out to be mandatory.

Another issue is the type of differentiation that PSCs must attain to be of therapeutic value. As already underlined, insufficient differentiation exposes unwanted *in situ* cell proliferation and uncontrolled *in situ* cell differentiation. However, excessive differentiation carries the risk that the injected cell preparation will fail to integrate the organ that must be repaired. For example, it is plain that terminally differentiated neurons displaying a full-grown axon will not be able to connect themselves with the surrounding or distant cells of the nervous system, hence diminishing the regenerative purpose of the cell injection. Furthermore, inappropriate differentiation such as a fetal phenotype to treat adult patients will prevent effective functional improvements from being achieved. However, the capability of cells to acquire a functional phenotype after transplantation should not be disregarded. Kroon *et al*[63] by using hESCs differentiated *in vitro* into pancreatic-like cells (similar to fetal 6-9 wk pancreatic tissue), showed that these cells develop *in vivo* into endocrine cells similar to pancreatic islets and protect mice against hyperglycemia.

Another concern with PSCs, a concern also associated with some other stem cell types, is that culture conditions may select for abnormal cell clones that harbor chromosomal or other genetic abnormalities[76]. These abnormalities are not random, and several teams have described the recurrent gain of extra copies of the long arm of chromosome 17, and the short arm of chromosome 12 or chromosome 20[77-80]. These karyotypic changes are similar to that of testicular germ cell tumors and may therefore raise safety concerns[79]. In addition to these chromosomal abnormalities, other changes have been described, including microarray comparative genomic hybridization (CGH) and promoter methylation[81]. The high proliferation rate of PSCs, metabolic stress in large cell colonies, and enzymatic passaging may contribute to these genetic and epigenetic changes acquired over the long term in *in vitro* culture. IPSCs bring further worry on the subject because some of the barriers to cell reprogramming are the same that prevent malignant transformation, namely the p53/mdm2/p21 and the Ink4/p16/Arf pathways[82-86]. Indeed, alleviating these barriers by genetic means results in a marked increase in cell reprogramming, but at the expense of DNA integrity. The team of Maria Blasco has reported that, during reprogramming, the presence of pre-existing, but tolerated, DNA damage resulted in the activation of a DNA damage response and p53-dependent apoptosis. This response was abrogated by p53 downregulation, producing iPSCs carrying persistent DNA damage and chromosomal aberrations[82]. Consequently, it will be mandatory to screen human iPSCs for genetic alteration just after reprogramming, as well as after prolonged *in vitro* cell culture as noted above.

The matter of allogenicity is raised by the fact that the probability of a given hESC to be HLA compatible with a patient is exceedingly low. There are several ways to resolve HLA disparity between cell lines and patients. One way is

to immunomodulate patients receiving HLA-incompatible cells. For certain organs, this has proven feasible, therefore it may be possible for hESCs that have been differentiated in certain cell types, but not for all. For example, complete HLA disparity precludes the injection of immune system cells into a patient, whatever the immunosuppressive drugs given. Alternatively, a limited collection of chosen hESC lines could cover, with an acceptable HLA compatibility, a majority of the population. About 150 hESC lines obtained consecutively would provide a beneficial match (defined as one HLA-A or one HLA-B mismatch only, HLA-DR being matched) or better for 37% of the general population in the United Kingdom or in Japan[87,88]. Furthermore, the selection of PSC lines homozygous for the HLA locus would lower the number of cell lines necessary for the bank. This could be obtained by parthenogenesis, which produces hESC lines that are homozygous for the HLA locus, except in the unlikely case where the meiotic recombination would take place in the middle of the locus. It has been estimated that, in the Japanese population, 55 randomly selected parthenogenetic hESCs could cover 80% of the patients with a match for HLA-A, HLA-B and HLA-DR[88]. By screening 24000 individuals, it would be possible to select 50 HLA homozygotes for the HLA-A, HLA-B and HLA-DR loci, from which 50 iPSC lines could be derived, which would match more than 90% of the patients[89]. Alternatively, the iPSC technology is a way to generate autologous PSCs for each patient, paving the way to personalized regenerative medicine[90]. However, even when the hurdles to the generation and differentiation of human iPSCs in GMP conditions are solved, the problem will remain concerning the time scale necessary to generate, amplify and qualify autologous iPSCs, in contrast with the urgency for some diseases to be treated, such as heart infarct, and the considerable cost of such personalized medicine.

Finally, the use of human PSCs in research and in regenerative medicine has spurred countless debates on the ethics of research on human embryos[5]. It has been proposed that iPSCs could solve the ethics around PSCs as they are generated without the need to destroy a human embryo. However, in addition to the fact that this technology is still in its complete infancy stage, necessitating that the reprogramming technology becomes GMP compliant and virus integration free, some ethical issues have arisen, such as the theoretical possibility of generating a human being that would be of 100% iPSC origin by tetraploid embryo complementation, as suggested by rodent experiments[91,92], or the differentiation of iPSCs into gametes that could then be fertilized, generating a human embryo[93,94].

CONCLUSION

In early 2009, a cellular therapy protocol based on hESCs had been agreed on by the US Food and Drug Administration (FDA)[95]. This protocol, conducted by Geron, based in Menlo Park, California, was a phase I safety study for spinal-cord injury. What was to be the first PSC-derived treatment was delayed in September 2009 after animal data revealed microscopic cysts growing around the injury site. Hence, this promising stem cell category still awaits its first use in human therapeutics. The reasons for the discrepancy between the huge expectation for disease treatment and the effective use of these cells in a clinical setting are the technical hurdles listed above. Much research is still needed to effectively resolve these problems. The recent advent of the iPSC technology has considerably boosted the PSC field and will therefore contribute to accelerate the advent of applications for PSC in curing human diseases. However, it must not be forgotten that though iPSCs strongly resemble hESCs, there are differences[96]. Therefore, research on hESCs and human embryos is mandatory to define the similarities and dissimilarities between these two cell types before envisioning the use of human iPSCs in regenerative medicine.

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REVIEW

Application of human umbilical cord blood-derived mesenchymal stem cells in disease models

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Abstract

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) are regarded as an alternative source of bone marrow-derived mesenchymal stem cells because collection of cord blood is less invasive than that of bone marrow. hUCB-MSCs have recently been studied for evaluation of their potential as a source of cell therapy. In this review, the general characteristics of hUCB-MSCs and their therapeutic effects on various diseases *in vitro* and *in vivo* will be discussed.

Key words: Regeneration; Immune modulation; Nursing effect; Mesenchymal stem cells

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INTRODUCTION

Mesenchymal stem cells (MSCs) are refined as undifferentiated cells that are capable of self renewal and differentiation into several cell types such as chondrocyte, adipocyte, osteocyte, myocyte and neuron-like cells[1,2]. MSC can be isolated from bone marrow, umbilical cord blood, adipose tissue, placenta *etc*. Although bone marrow (BM) has been regarded as a major source of MSC, umbilical cord blood has recently been regarded as an alternative source for isolation of MSC[3,4]. Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have a capacity similar to that of BM-MSCs for multi-lineage differentiation[5]. In addition, hUCB-MSCs also possess activities for immune modulation, tumor tropism and nursing effect[6,7]. When compared with other MSCs from various sources, hUCB-MSCs could be considered a fascinating source for use in stem cell therapy. In this review, we introduce the general characteristics of hUCB-MSCs and its application in various disease models.

GENERAL CHARACTERISTICS OF hUCB-MSCS

Immunogenic phenotypes of hUCB-MSCs

Immune rejection in recipient patients is the primary issue associated with use of MSCs as an allogeneic cell source for cell based therapy involving transplantation. In fact, previous studies of the properties of immune-privilege have been carried out primarily in BM-MSCs where the surface immunogenic markers were hypo-immunogenic which may prevent proliferation of allogeneic lymphocytes[8-10]. In the same fashion as BM-MSCs, major histocompatibility complex-Ⅱ class molecules and costimulatory molecules, such as CD40, CD40 ligand, CD80 and

CD86 which are involved in T cell activation response for transplant rejection, are not expressed in hUCB-MSCs even when mitogenic or allogeneic stimulated signals are delivered. In addition, differentiation of hUCB-MSCs into chondrocyte or neuron-like cells did not elicit expression of these immunogenic surface molecules and could not provoke allocative lymphocyte proliferation in mixed lymphocyte reactions (MLR) *in vitro*[11-13]. Compared with BM-MSCs, hUCB-MSCs showed lower immunogenicity than BM-MSCs because of primitive characters originating from UCB. Indeed, undifferentiated or differentiated hUCB-MSCs can be successfully transplanted for cell based therapy due to permission of a greater degree of HLA mismatch without graft versus host disease but not in BM-MSCs. Therefore, immunogenic phenotypes of hUCB-MSCs can retain low immunogenicity under certain biological conditions which provide advantages for development of off-the-shelf products for clinical application of cell transplantation. Thus, hUCB-MSCs show promise as a source for stem cell therapy.

Immune regulation properties of hUCB-MSC

MSCs are known to have immune suppressive action on lymphocyte proliferation in MLR by alloantigen and mitogens such as phytohemagglutinin and to reduce the level of proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Recent evidence has demonstrated that hUCB-MSCs can suppress not only the function of mature dendritic cells but also increase the portion of regulatory T cells related to immune regulation[12,13]. This regulation of immune response by MSCs is mediated by soluble factors and cell to cell contact mechanisms. At present, several soluble factors involved in immune suppression have been reported including transforming growth factor-β (TGF-β)[14]. However, induction of these cytokines was not observed under conditions of immune suppression by hUCB-MSCs and study of contact dependent inhibition by hUCB-MSCs is in progress. In fact, based on our unpublished data, hUCB-MSCs elevated the level of prostaglandin E2 and induced indoleamine 2, 3-dioxygenase (IDO). In addition, the surface molecule HLA-G which is involved in immune tolerance in pregnancy was detected in hUCB-MSCs by fluorescence activated cell sorter analysis. It has been suggested that the molecular mechanism(s) or strategy for immune regulation by MSCs is dependent on species and tissue origin[15,16].

Consequently, to understand and utilize the immune regulation properties of hUCB-MSCs for application in the treatment of a number of human immunological diseases, the molecular mechanism underlying the immune modulatory functions of hUCB-MSCs should be further investigated.

APPLICATION OF hUCB-MSCS IN DISEASE MODELS

hUCB-MSCs for cartilage regeneration

Since MSCs are capable of differentiation into mesodermal origin, several groups have tried cell therapy for osteoarthritis (OA), the most common type of arthritis. Two representative common diseases of cartilage degeneration include OA and rheumatoid arthritis (RA). OA is a progressive degenerative disease of the cartilage that is induced by complex factors that include increasing age, mechanical stress and inflammation leading to primary focal cartilage degradation and its functional loss. RA is a chronic autoimmune disease characterized by inflammation of the lining of the synovium or joints that causes long term joint damage, particularly in cartilage[17]. MSCs are known for differentiation into mesodermal derived tissue such as cartilage, bone, adipose and muscle. In fact, hUCB-MSCs have much higher chondrogenic differentiation potential among mesodermal differentiation potentials which might lead to regeneration of damaged cartilage. In addition to this chondrogenic differentiation potential of MSCs, recent advances in our understanding of the regeneration mechanism for cartilage defects have demonstrated that MSCs also show potent immunosuppression and anti-inflammatory effects[11-14]. These properties might be due in part to specific secreted factors, including some types of cytokines and growth factors. For instance, it has been reported that thrombospondin-1, 2 (TSP-1, 2) functions as an anti-inflammatory factor in RA by suppressing production of proinflammatory mediators such as IFN-γ and TNF-α, inducing depletion of synovium residing T cells and reducing infiltration of monocytes/macrophages in articular tissues[18,19]. In this fashion, chondrogenic differentiation and paracrine actions might be involved in replacement of damaged cartilage tissues and stimulation of the regeneration process.

However, several research teams have conducted studies to demonstrate the therapeutic potential of BM-MSCs for OA or RA[20-24] but few studies have reported on hUCB-MSCs. Despite growing experience and knowledge of these attempts, the molecular mechanisms underlying cartilage repair and regeneration by MSCs remain uninvestigated. Therefore, extensive studies of hUCB-MSCs therapeutic mechanisms are required for an understanding of their regenerative potential and for efficient and safe clinical application. Fortunately, supported by our clinical trial phase Ⅰ / Ⅱ results (NCT01041001), we believe that adult stem cell therapy using hUCB-MSCs for cartilage degenerative disease is a promising alternative to previous treatments if optimal hUCB-MSCs therapy conditions are adjusted by a full understanding of the important regeneration mechanism for diseases of cartilage degeneration.

hUCB-MSCs for glioma

Interestingly, MSCs have been shown to migrate toward glioma[25]. This phenomenon could be applied to tumor therapy with MSCs loaded tumor therapeutic agent. TRAIL, IL-12, IFN-β and cytosine deaminase have been used as therapeutic agents in MSCs-mediated delivery. In particular, TRAIL-secreting hUCB-MSCs showed therapeutic effects in an intracranial glioma model[26]. Injection of engineered MSCs inhibited tumor-growth and prolonged the lifespan of glioma-bearing mice compared

with control. Although this glioma tropism has been demonstrated, the exact molecular mechanism has not been elucidated. As glioma over expressed interleukin-8 (IL-8), our group tested IL-8 action in hUCB-MSCs migration toward glioma. Interestingly, a high level of IL-8 was detected in the conditioned media of co-cultured glioma cells with hUCB-MSCs. Recombinant IL-8 treatment of hUCB-MSCs enhanced migration to the lower chamber under the Transwell system. This effect was reduced by pre-treatment of hUCB-MSCs with antibody against CXC chemokine receptor 1 (CXCR1) and CXCR2, IL-8 receptor. Therefore, IL-8 will be an attracting factor for hUCB-MSCs migration toward glioma[27]. We expect that IL-8 receptor over expression in hUCB-MSCs will efficiently deliver cancer drugs to glioma. For application of hUCB-MSCs in human glioma, more efficient and safe methods of therapeutic gene expression in hUCB-MSCs should be established.

hUCB-MSCs for ischemic brain damage

Because brain injury due to ischemia cannot be recovered and can result in severe functional defects in the brain, stroke is a primary disease target for stem cell therapy. In ischemia studies, most data show that cell therapy is performed using hUCB. The first evidence of a therapeutic effect of hUCB came from Chopp's laboratory, where rat was used for middle cerebral artery occlusion to induce focal ischemia. Intravenous administration of hUCB reduces behavioral deficits after stroke in rats[28]. Recently, infarct volume was reduced 1 d after intra arterial delivery of hUCB-MSCs in canine cerebral ischemia whereas infarct volume was increased in the control groups. Transplanted hUCB-MSCs were differentiated into neurons and astrocytes in and around endothelial cells and secreted brain-derived neurotrophic factor and vascular endothelial growth factor at 4 wk after transplantation[29]. Jeong *et al*[30] reported that transplantation of hUCB-MSCs into contralateral regions of injured rat brain at 7 d after injury resulted in significant behavioral improvement. In addition, PKH26-labled hUCB-MSCs differentiated into neural cells at the injured site at 4 wk after transplantation. These results suggested that transplantation of hUCB-MSCs could be used in clinical trials for ischemia. Despite these interesting data, stem cell therapy using hUCB-MSCs have to make critical decisions with regard to the route of transplantation, type of injected cell (hUCB *vs* hUCB-MSCs) and timing of transplantation.

hUCB-MSCs for lung diseases

Progression of acute respiratory distress syndrome is demonstrated by loss of lung tissue as a result of inflammation and fibrosis. Human umbilical cord cells derived from Wharton's jelly with a phenotype consistent with that of MSCs (uMSCs) were treated using a bleomycin induced-lung injury mouse model[31]. After 2 wk, systemic administration of uMSCs was located in the area of inflammation and fibrosis. Injected uMSCs reduced inflammation and inhibited expression of TGF-β, IFN-γ and proinflammatory cytokines, including macrophage migratory inhibitory factor and TNF-α. Furthermore, collagen level was decreased due to up-regulation of matrix metalloproteinase-2 and reduced endogenous inhibitors, tissue inhibitors of matrix metalloproteinases. These results suggested that uMSCs participate in anti-fibrosis in lung injury. Interestingly, hUCB-MSCs transplantation can attenuate hyperoxia-induced lung injury in immunocompetent newborn rats[32]. A single dose of PKH26 labeled hUCB-MSCs was administered intratracheally (2×10^6 cells) at postnatal day 5. Two abilities of hUCB-MSCs, immune modulation and differentiation potential, were evaluated after hUCB-MSCs administration. The hyperoxia-induced increase in the number of dead cells, myeloperoxidase activity, abnormal alveolarization and level of IL-6 mRNA were significantly decreased with intratracheal hUCB-MSCs administration. Furthermore, increased level of TNF-α, TGF-β mRNA, α-SMA protein and collagen were significantly reduced by hUCB-MSCs. As pKH26-labeled differentiated lung epithelial cells were observed in damaged lung, collectively, hUCB-MSCs could be used for cell therapy *via* both anti-inflammation and regeneration in hypoxia induced lung injury.

hUCB-MSCs for liver diseases

Cirrhosis is a consequence of chronic hepatic injury characterized by replacement of liver tissue by fibrosis and scar tissue. Cirrhosis is most commonly caused by alcoholism, fatty liver and hepatitis B and C. No effective therapy is currently available for this disease[33]. Recent reports have shown that MSCs have the capacity for differentiation into hepatocytes. In carbon tetrachloride (CCl_4)-induced cirrhosis in a rat model, hUCB-MSCs infusion showed inhibition of TGF-β1, collagen type I and α-SMA expression. In addition, CM-DiI-labeled hUCB-MSCs expressed hepatocyte-specific markers, human albumin and α-fetoprotein in injured liver[34]. Similar data was observed by Yan *et al*[35]. Interestingly, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling and proliferating cell nuclear antigen staining showed that transplanted hUCB-MSCs could prevent hepatocyte cell death and stimulate proliferation. According to these data, hUCB-MSCs could be useful in liver therapy. Liver contains endogenous abundant progenitor cells for recovery of liver damage. Therefore, it is currently difficult to determine which stem/progenitor cell populations are best for liver disease therapy.

CONCLUSION

In the effort to overcome incurable disease, stem cell therapy has been regarded as the next solution. In particular, adult stem cells such as hUCB-MSCs have shown therapeutic efficacy in various animal disease models. Compared to embryonic stem cells, adult stem cells have several advantages for use in stem cell therapy. Adult stem cells are relatively free of ethical issues, immune rejection and tumor formation. In particular, hUCB-MSCs are obtained from discarded umbilical cord blood after child birth. If informed consent is available from pregnant mothers,

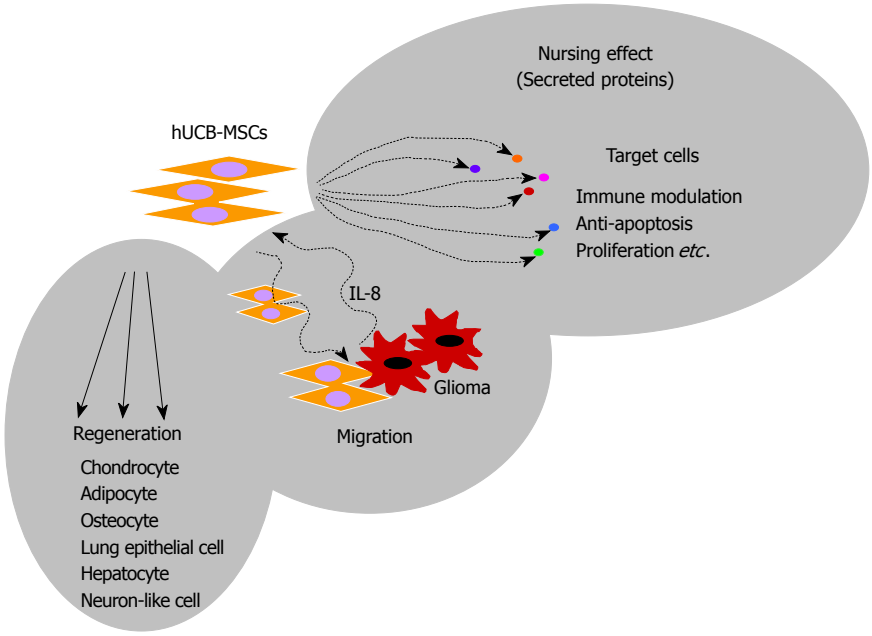

Figure 1 Multifunctional therapeutic activities of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). Transplanted hUCB-MSCs participate in cartilage, lung and liver regeneration through differentiation of damaged tissues as well as attenuation of inflammation and apoptosis. Interestingly, hUCB-MSCs can migrate toward glioma. When migrated hUCB-MSCs express apoptotic gene such as TRAIL in glioma region, tumor size will be decreased by secreted TRAIL from hUCB-MSCs.

hUCB-MSCs can be easily isolated from cord blood under aseptic conditions. Since public and private cord blood banking systems are well established in Korea, basic and clinical investigation into development of cell therapies has been intense. Furthermore, immunological safety has been proven in several journals although application of hUCB-MSCs is based on allogeneic transplantation. In addition, there have been no reports of tumor formation after transplantation of hUCB-MSCs in animals or humans. From this review, we could conclude that regeneration of target tissue by differentiation and nursing effect by secreted proteins are among the molecular mechanism of hUCB-MSCs action in various diseases (Figure 1). In the early days of stem cell research, regenerative medicine was the main stream of research. However, paracrine action of stem cells under pathological conditions is now emerging. The study of hUCB-MSCs is incomplete compared to that of BM-MSCs. However, once basic scientific understanding of hUCB-MSCs have shown further advancement and conditions for isolation and expansion of hUCB-MSCs are well established for the clinic, hUCB-MSCs will be a fascinating source for use in stem cell therapy.

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Events Calendar 2010

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Stem Cells 2010
Crowne Plaza Hotel, St James, London, United Kingdom

May 11-13, 2010
World Stem Cells and Regenerative Medicine Congress
London, United Kingdom
http://www.terrapinn.com/2010/stemcells/index.stm

May 12-15, 2010
Stem Cells, Tissue Homeostasis and Cancer
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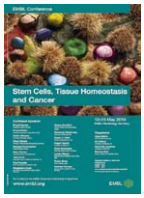

June 16-19, 2010
ISSCR 8th Annual Meeting
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http://www.isscr.org/meetings/index.cfm

June 27-July 2, 2010
The 9th Gordon Conference on Cell Biology of the Neuron
Waterville Valley Resort, NH, United Kindom

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http://www.stemcellcongress-dresden.org/

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The 2010 Gordon Conference on Cell Death
Salve Regina University, Newport, RI, United Stated

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer

disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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