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FIELD OF VISION

Disagreements in the therapeutic use of mesenchymal stem cell-derived secretome

Ferenc Sipos, Györgyi Műzes

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Abstract

In a recent article, the authors provide a detailed summary of the characteristics and biological functions of mesenchymal stem cells (MSCs), as well as a discussion on the potential mechanisms of action of MSC-based therapies. They describe the morphology, biogenesis, and current isolation techniques of exosomes, one of the most important fractions of the MSC-derived secretome. They also summarize the characteristics of MSC-derived exosomes and highlight their functions and therapeutic potential for tissue/organ regeneration and for kidney, liver, cardiovascular, neurological, and musculoskeletal diseases, as well as cutaneous wound healing. Despite the fact that MSCs are regarded as an important pillar of regenerative medicine, their regenerative potential has been demonstrated to be limited in a number of pathological conditions. The negative effects of MSC-based cell therapy have heightened interest in the therapeutic use of MSC-derived secretome. On the other hand, MSC-derived exosomes and microvesicles possess the potential to have a significant impact on disease development, including cancer. MSCs can interact with tumor cells and promote mutual exchange and induction of cellular markers by exchanging secretome. Furthermore, enzymes secreted into and activated within exosomes can result in tumor cells acquiring new properties. As a result, therapeutic applications of MSC-derived secretomes must be approached with extreme caution.

Key Words: Mesenchymal stem cells; Secretome; Exosomes; Regeneration; Therapy; Cancer

Core Tip: The authors of a recent article provide a detailed summary of the properties and biological functions of mesenchymal stem cell (MSC)-derived exosomes, one of the most important fractions of the MSC-derived secretome. However, in addition to their undeniable benefits, there are a number of risks associated with their use. Exosomes have the potential to have a significant impact on the development of diseases such as cancer. The use of MSC-derived secretomes for therapeutic purposes must be approached with extreme caution.

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INTRODUCTION

Commentary on hot topics

Stem cell and tissue engineering studies appear to be critical components of regenerative medicine. Stem cells are characterized as totipotent, pluripotent, multipotent, or unipotent depending on their ability to differentiate into new cell lines. While allogeneic cells can create complications such as immunological rejection, when autologous cells are utilized, rejection can be avoided, making this a less risky mode of treatment.

Adult stem cells, such as mesenchymal stem cells (MSCs) and hematopoietic stem cells, are the most commonly used types in clinical practice, owing to their availability from individuals with various medical conditions (*e.g.*, aplastic anemia, Duchenne muscular dystrophy, ankylosing spondylitis, *etc.*) [1].

MSCs have the ability to self-renew while also possessing a limited potential to distinguish from one another. Bone marrow, adipose tissue, liver, skin, lungs, cord blood, and fallopian tubes are their primary sources[2].

MSC-based treatments are widely used around the world, with their effects mediated *via* induced differentiation, immunological modulation, cell fusion, paracrine actions, mRNA or micro-RNA (miRNA) carriage, and mitochondrial metastasis. MSCs for therapeutic purposes face challenges such as maintaining a homogeneous culture and, further, characterization of the cells[3]. In addition to cell replacement, MSCs possess a diverse array of functional characteristics (*i.e.*, angiogenesis, fibrosis inhibitory as well as anti-apoptotic capacity, directed migration, immunomodulation, growth and differentiation supporting activity on other stem cells)[4-7]. The release of bioactive components, referred to as the secretome, into the conditioned media of cell culture is one of their most intriguing qualities[8]. The secretome is composed of two fractions: Soluble and vesicular. Immunomodulatory molecules, chemokines, cytokines, and growth factors are abundant in the soluble fraction. The vesicular fraction consists of extracellular vesicles that can be categorized as apoptotic bodies, microvesicles, and exosomes based on their diameter and synthesis route. Exosomes and microvesicles containing lipids, proteins, or nucleic acids comprise the secretome derived from MSCs[8]. As indicated above, the secretome has the potential to directly stimulate target cells through endocytosis and to exert a wide range of actions[9]. However, it is critical to keep in mind that, depending on where the MSCs come from, the secretome's therapeutic potential may differ[10].

MSCs are an important pillar in regenerative medicine due to their wide range of functional capabilities. As a result, to ensure that no functional or genetic alterations occur during clinical use, their biosafety characteristics should be examined. MSCs have a number of disadvantages, including their detrimental effect on the pulmonary microvasculature, host cell rejection, and ectopic tissue formation [11-13]. Additionally, it has been demonstrated that MSCs have a very limited capacity for regeneration, particularly in pathological conditions. While MSCs are found in a variety of tissues, their numbers are relatively small. Furthermore, transplanted cells' viability and uptake into host tissues are frequently compromised[14]. Also, a variety of factors, such as the donor's age, the number of passages and culture conditions used during *in vitro* growth, administration procedure, and the deleterious host microenvironment encountered by the relocated MSCs, may have a negative effect on the cells' proclivity for survival and engraftment in host tissues[15]. Recent studies have also indicated possible pro-tumorigenic activities of MSCs[16,17], along with pro-fibrogenic and pro-coagulant potentials[18,19], a higher risk of infections (*e.g.*, zoonotic illnesses) during the *in vitro* growth process[20], and the unfavorable heterogeneity of their differentiation potential (Figure 1)[21]. Due to these drawbacks, their clinical application has been limited. As a result, it is necessary to develop alternative, complication-free MSC-based therapeutic strategies.

In a recent review by Ma *et al*[22], the authors provide a detailed summary of the characteristics and biological functions of MSCs and discuss the potential mechanisms of action of MSC-based therapies.

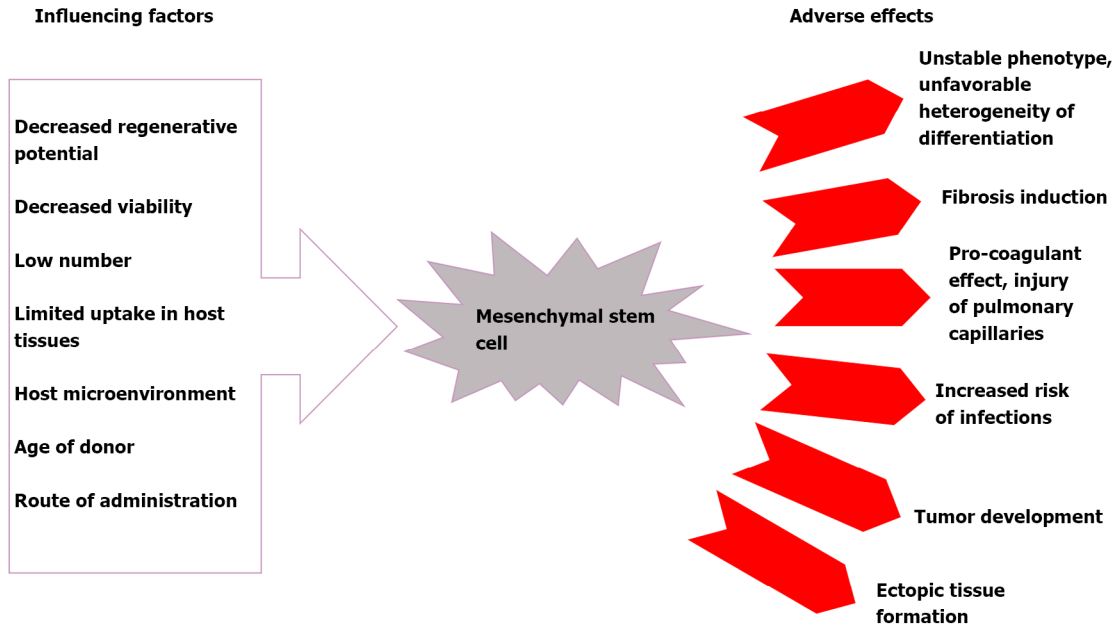

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Figure 1 Factors influencing the therapeutic potential of mesenchymal stem cells and their consequences.

They describe the morphology, biogenesis, and current isolating techniques of exosomes, one of the most important fractions of the MSC-derived secretome.

UNDESIRABLE EFFECTS OF THE MSC SECRETOME

The consequences of the treatments with MSC-derived cells have heightened interest in the MSCs' secretome for therapeutic purposes. The application of MSCs' secretome has a number of significant benefits, including the complete absence of the necessity for an invasive solution to obtain cells, the capability of conducting pharmacological dosage and safety tests, the convenience of application, and the possibility of manipulating the composition[23]. Soluble and vesicular factors derived from MSCs exhibit a variety of unique properties that may make them a precious tool for therapeutic reasons[8]. Ma *et al*[22] compiled a list of the numerous regenerative medicine benefits of MSC-derived exosomes[22]. Simple collection, long-term stability, safety, optimal drug transport capacity, and tissue or microenvironment-specific targeting are the most critical of these. Additionally, they summarized recent research on the actions of MSC-derived exosomes in different diseases affecting the skin, bone, muscle, kidney, cardiovascular system, liver, and nervous system.

However, practical difficulties appear in cases of those entities, as their physical and biochemical properties frequently cause complications to obtain them as perfect and correctly characterized preparations. As a result, the International Society for Extracellular Vesicles developed guidelines for the field in 2014 (*i.e.*, Minimal Information for Studies of Extracellular Vesicles), which were recently revised in 2018[24].

We must not forget that exosomes can also play a significant role in the development of diseases such as cancer. When tissue is damaged, MSCs are recruited to aid in the repair and regeneration of wounds. Also, aggressive tumor development results in inflammation-related tissue injury as a result of intense cell recruitment and cross-modulation. By exchanging secretome, MSCs have the potential to interact with tumor cells[25-28], promoting reciprocal interchange and induction of biological markers[29,30].

Not only the direct effect of the MSC-secreted soluble fraction, but enzymes excreted into and activated inside exosomes (primarily matrix metalloproteinases and their regulators) could make malignant cells have novel properties[25]. The secretome's vesicular fraction is involved in the formation of the pre-metastatic niche and tumor neovascularization. In addition, abnormalities in the extracellular matrix may influence cancer progression by promoting fibroblastic switching and acquisition of mesenchymal mode[26].

The incorporation of MSC-derived exosomes has been linked to the development of ecto-5'-nucleotidase activity in a subset of tumor cells[25]. Tumor cells equipped with this unique ability are capable of suppressing and modulating inflammation-inducing activity by way of the stimulation of adenosine receptor signaling located in the external membrane of the majority of immunocompetent cells, (*e.g.*, tumor-infiltrating T-cell function)[31,32].

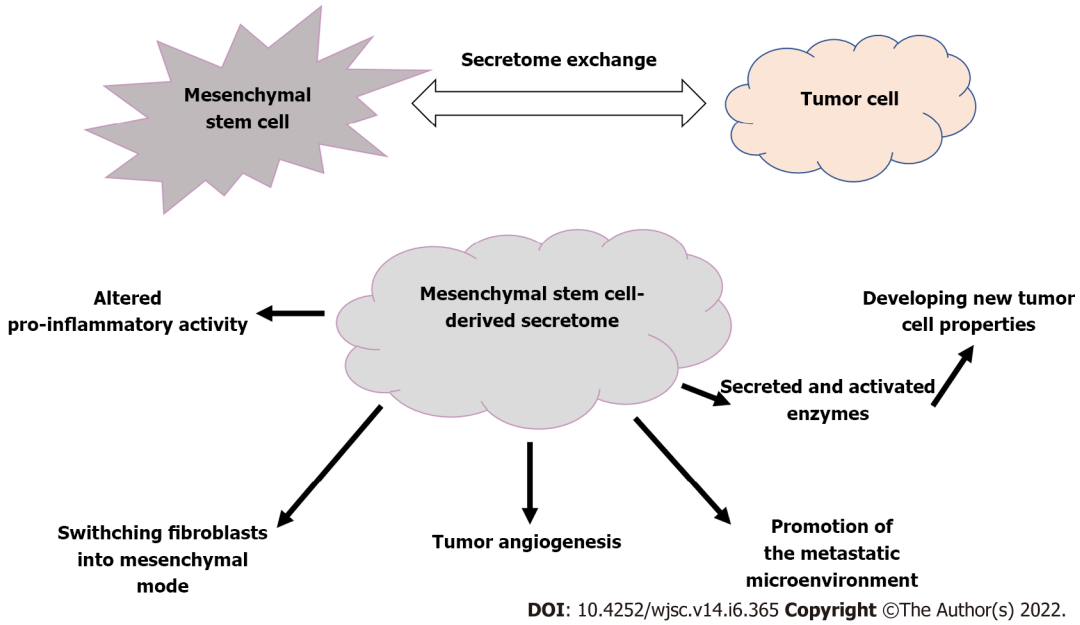

Figure 2 The secretome exchange between mesenchymal stem cells and tumor cells has unfavorable effects.

In the opposite direction, tumor cells can also affect and modify MSCs through the use of their secretome[22,26]. Extracellular vesicles produced by cancer stem cells are capable of establishing a metastasis supportive compartment and inducing an epithelial to mesenchymal transition, allowing tumors to spread more easily (Figure 2)[26].

Along with undesirable biological properties, current methods for isolating the vesicular secretome (*e.g.*, membrane filtration, ultracentrifugation, precipitation, immunoaffinity capture technology, and size exclusion chromatography) are inefficient, yielding small quantities of low-purity, occasionally distorted extracellular vesicles. As a result, their further application presents difficulties[22,33-35].

CONCLUSION

In accordance with ClinicalTrials.gov, the number of studies utilizing the MSC-derived secretome is fairly small (*i.e.*, ten), notwithstanding the fact that just three have been completed so far. While the restorative potential of MSC-originated secretome appears auspicious, care is advised. Not only is the content and function of the secretome formed from MSCs largely dependent on the environment from which they were derived (*i.e.*, healthy, inflammatory or tumorous environment), but the therapeutic targeting of the secretome is also difficult at the moment[36]. Whichever method of application is employed, it is not yet feasible to be assured that the biologically active chemical will work on a particular cell type, nor is it totally likely to identify how the intended physiological action of the secretome is altered by the surrounding milieu.

Currently, we also lack knowledge on how drug combinations used in disease conditions affect MSCs and their secretome. By altering MSCs to carry anticancer miRNAs, oncolytic viruses, and anticancer drugs into tumor areas, scientists are able to overcome a number of barriers[37]. However, additional research is required to determine the influence of probable epigenetic or genetic alterations in MSCs on the content and biological functions of the secretome. This is critical to prevent the possibility of tumorigenicity[38].

Along with the technical challenges associated with locating and separating MSCs, laboratory approaches that are novel and efficient are required to extract the MSC-derived secretome in sufficient quality and quantity for application in daily routines. In addition, it would be advantageous to minimize the time and expense involved in these novel procedures, thereby effectively promoting their spread. In conclusion, there is no doubt that, in relation to cell-based techniques, cell-free bioactive components such as the secretome could serve as a significant option in translational medicine.

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REVIEW

Adipose tissue in bone regeneration - stem cell source and beyond

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Abstract

Adipose tissue (AT) is recognized as a complex organ involved in major homeostatic body functions, such as food intake, energy balance, immunomodulation, development and growth, and functioning of the reproductive organs. The role of AT in tissue and organ homeostasis, repair and regeneration is increasingly recognized. Different AT compartments (white AT, brown AT and bone marrow AT) and their interrelation with bone metabolism will be presented. AT-derived stem cell populations - adipose-derived mesenchymal stem cells and pluripotent-like stem cells. Multilineage differentiating stress-enduring and dedifferentiated fat cells can be obtained in relatively high quantities compared to other sources. Their role in different strategies of bone and fracture healing tissue engineering and cell therapy will be described. The current use of AT- or AT-derived stem cell populations for fracture healing and bone regenerative strategies will be presented, as well as major challenges in furthering bone regenerative strategies to clinical settings.

Key Words: Adipose tissue; Bone metabolism; Fracture healing; Adipose-derived stem cells; Multilineage differentiating stress-enduring; Dedifferentiated fat cells; Bone engineering

Core Tip: Adipose tissue (AT) is a multifunctional organ with intricate body functions. Different AT compartments have complex interrelations with bone metabolism, tissue maintenance and fracture healing. AT-derived stem cell populations are promising tools for bone regeneration. The current use of AT- or AT-derived stem cell populations for fracture healing and bone regenerative strategies will be presented, as well as major challenges in furthering bone regenerative strategies to clinical settings.

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INTRODUCTION

Adipose tissue (AT) has multiple roles in body energy balance, regulation of food intake, immunomodulation and growth and functioning of the reproductive organs[1]. In recent years, the understanding of AT functioning has evolved from considering it a lipid storage, cushioning and thermal insulating mass to its recognition as the largest endocrine organ within the mammalian body[2]. AT-derived signaling molecules, adipokines and cytokines have a crucial role in local and systemic regulation by controlling energy expenditure, glucose homeostasis, insulin metabolism and immune cell function to support cell proliferation in normal and pathological states. AT has received increased attention in recent years mainly due to its abnormal expansion in obesity and metabolic syndrome. Normal AT has intricate roles in maintaining healthy body functioning. Not surprisingly, obesity is very often accompanied by many endocrine and metabolic disturbances, such as insulin resistance, type 2 diabetes mellitus (T2D), disorders in immune regulation and response to pathogens, and tumor occurrence, progression and metastasis[3]. The pathological lack of AT (congenital, human immunodeficiency virus - or age-related lipodystrophy) is linked to multiple metabolic and immune abnormalities, such as insulin resistance, liver steatosis and dyslipidaemia[4]. Given its multifaceted roles in controlling body homeostatic mechanisms, AT involvement in tissue and organ healing and regeneration is complex and only partially recognized. Understanding AT involvement in regeneration and repair is a relatively new concept. Consistent research in recent decades has focused on AT as a mesenchymal stem cell source. Pluripotent stem cells extracted from white AT (WAT) under special conditions together with transdifferentiated adipocytes have the potential to accelerate progress in the field of bone engineering. AT, with its complex paracrine and endocrine signaling and angiogenetic potential, might also be used to support the bone regenerative niche. The types of AT - WAT, brown (and beige) AT (BAT) and bone marrow AT (BMAT) - will be very briefly introduced with emphasis on BMAT given its direct involvement in bone metabolism. Current strategies that employ AT or AT-derived cell populations for fracture healing or bone regeneration will be presented.

WAT IS A COMPLEX ENDOCRINE ORGAN

In humans, WAT is formed starting from the second semester of gestation and continues throughout life, even in adults[5]. In mice, WAT adipocytes are derived from mesenchymal progenitors within the somites or lateral plate mesoderm, which could be the case for humans[6], except for minor fat deposits of the skull derived from the ectodermal neural crest[7]. WAT therefore shares a developmental origin with all the components of connective tissue (muscle, bone, tendon and fascia). WAT is composed of mature cells (adipocytes) that contain unilocular deposits of lipids (triacylglycerol) occupying up to 90% of the cytoplasm. Only one-third of the tissue is represented by mature adipocytes, and other cellular components are preadipocytes, stromal cells, mesenchymal progenitors and immune components [monocytes and macrophages (Mcfs)]. WAT represents the major energy storage system of the body and is the main lipid deposit[8]. It has a mechanical role in thermal insulation, organ cushioning and protection from trauma. In humans, mature adipocytes store lipids synthetized in the liver and, to a minor extent, within AT itself by lipogenesis, an insulin-dependent enzymatic process. Fatty acid (FA) and triglycerides (TG) availability in other organs in the case of energy demand are dependent on the activity of AT lipolytic enzymes[9]. Two main compartments of WAT are described based on their anatomic location: Subcutaneous and visceral. Their characteristic distribution is sex hormone-dependent with visceral compartment testosterone and subcutaneous oestrogen - controlled[10]. Mature adipocytes release bioactive molecules (commonly denominated adipokines) that exert paracrine and endocrine functions in maintaining body energetic metabolism, insulin sensitivity, food intake, immune modulation, haematopoiesis, bone metabolism, angiogenesis, coagulation and fibrinolysis. Adipokines are a set of cytokine (leptin, adiponectin visfatin) chemokine (nitric oxide, hydrogen peroxide) growth

factors, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and colony-stimulating factor 1 complement factors, such as adipsin, B, C, and C3[11]. It is beyond the scope of this paper to describe their role in body balance; however, it should be noted that numerous bioactive molecules released by normal mature adipocytes are among crucial factors implicated in all the stages of wound and bone healing.

WAT is a very dynamic tissue that largely fluctuates in quantity but also its functional qualities across growth, maturity and ageing, physiological stages and diseases. Obesity as a disease of excess and lipodystrophy as a sum of conditions where WAT does not form or becomes atrophic are both associated with important perturbations in adipocyte quality and functioning. In obesity, adipocytes increase in number (hyperplasia) and size (hypertrophy), while in lipodystrophy, *de novo* adipogenesis and lipid droplet formation are impaired. Obesity and lipodystrophy are accompanied by severe metabolic disorders, such as hypertriglyceridemia, insulin resistance, diabetes, and fatty liver, as well as by severe perturbation in adipokine release. Impaired metabolic status and abnormal WAT paracrine and endocrine signalling in diseases of lack and excess have consequently impaired wound and bone healing.

ROLE OF WAT IN TISSUE REGENERATION

WAT deposits and healthy functioning mature adipocytes are involved in homeostatic maintenance, turnover and repair of several organs and tissues, such as hair follicles, skin and mammary glands[12]. Bone morphogenetic protein (BMP) expression by mature adipocytes may be regulators of the quiescent stage of hair follicle stem cells and supportive of hair lineage specification and differentiation during hair growth[13]. Adipogenic progenitors (AP) within dermal WAT stimulate hair stem cell follicle activation. Intradermal AP injection was shown to increase the growth of hair cell follicles, while leptin expressed by mature adipocytes induced the activation of hair cell follicles and hair shaft growth[14]. Mature adipocytes are a major component of the dermis that supports the skin epithelial layer and keratinocytes. Dermal WAT was previously thought to exert a cushioning and insulating function; however, its role in supporting skin integrity and promoting wound healing is increasingly recognized and explored. Mature adipocyte-secreted adiponectin and leptin were shown to increase keratinocyte proliferation *in vitro* and to consistently increase wound re-epithelialization in mouse models[15], while adiponectin-deficient mice suffer from severely delayed wound epithelialization[16]. Adiponectin regulates local apoptosis, and its absence in diabetic subjects might explain hyperkeratosis and thickened wound margins characteristic of chronic ulcers[17]. Another adipokine, leptin, was shown to increase re-epithelialization, angiogenesis and wound contraction after injury[18]. Functional mature adipocytes are required for the third stage of wound healing and extracellular matrix (ECM) deposition by fibroblasts. Mouse strains that lack mature adipocytes have impaired fibroblast repopulation during wound healing, and incomplete ECM deposition leads to recurrent wounding in this model[19]. Mature adipocytes are required for the development of a functional mammary gland ductal tree. In lipodystrophic and inducible adipocyte loss mouse strains, mammary gland formation is impaired[20].

WAT presence within muscles is commonly associated with tissue degeneration. Muscle fatty atrophy is a frequent clinical correlation with insulin resistance and increased body max index (BMI). However, if mature adipocytes in muscle tissue are a witness of impaired muscle function, common adipogenic and fibroblast progenitor (FAP) multipotent muscle resident stromal cells are required for muscle wound healing and tissue growth. FAPs enhance the rate of differentiation of primary myogenic progenitors *in vitro* and expand during muscle injury. FAPs were shown to supply transient pro-differentiation signals for proliferating myogenic progenitor cells after muscle injury in animal models[21]. Interestingly, when FAPs are transplanted within subcutaneous or dermal tissue, they differentiate into WAT, demonstrating the role of the environment in their activation and differentiation. FAPs are present in human skeletal muscle tissue and could be the source of fatty infiltration during muscle degeneration[22]. These progenitors have dual responsiveness to environmental cues, being able to generate either adipocytes or to support muscle hypertrophy. FAPs respond to metabolic stress during metabolic disorders by conversion to adipocyte lineage, as well as mechanical stress, during physical activity by fibrogenic conversion and contribution to satellite cell activation. This response might explain their opposite role in fatty infiltration and muscle healing and hypertrophy[23].

WAT IN BONE METABOLISM AND FRACTURE HEALING

WAT and bone metabolism are coordinated directly by the central nervous system through sympathetic and parasympathetic innervation and indirectly by circulating hormones. Sympathetic innervation controls WAT metabolism, while the parasympathetic role in this tissue is less agreed upon. Indirectly, sympathetic innervation of adrenal glands controls glucocorticoid release. Elevated levels of glucocorticoids are clinically associated with bone loss and hypertrophic WAT expansion during obesity[24]. Ghrelin, a neuroendocrine hormone produced in the gastrointestinal tract, has a dual role in regulating

white adipocyte metabolism by increasing lipoprotein lipase and FA synthase and increasing peroxisome proliferator-activated receptor-β (PPAR-β), stimulating the synthesis of TG and their mobilization. Ghrelin was found to directly promote osteoblast proliferation and differentiation, resulting in increased bone mineral density (BMD) in animal models[25]. Several WAT-released adipocytes have dual roles in bone and adipose maintenance and turnover. Leptin is known to inhibit food intake, increase energy expenditure and reduce WAT by increasing lipolysis. Leptin has a direct effect in promoting bone marrow stem cell (BMSC) differentiation to osteoblasts and preventing their adypogenic conversion[26]. Leptin increases the expression of osteocalcin (OC), alkaline phosphatase and collagen I, which are required for osteoblast maturation[27]. Leptin is involved in controlling bone resorption by increasing osteoclast-inhibiting osteoprotegerin (OPG)[28]. Direct administration was found to increase BMD and femur length in leptin-deficient mice[29]. Adiponectin, the adipokine that is most commonly found in plasma, improves insulin sensitivity, increases the rate of FA oxidation and reduces inflammation and fatty muscle infiltration. Low levels of circulating adiponectin are found in obese and lipodystrophic mouse models[30], have been implicated in inducing insulin resistance in obese subjects and proposed as a serum biomarker for detecting metabolic syndrome[31]. Adiponectin was found to promote osteogenesis in BMSCs by indirectly increasing BMP2 expression[32], to increase alkaline phosphatase (ALP), collagen I and OC expression and to promote osteoblast proliferation and differentiation in a dose-dependent manner[33]. Similar to leptin, adiponectin inhibits osteoclast activity through distinct mechanisms. Adiponectin decreases the expression of cathepsin K and acid-resistant phosphatase, which are osteoclast regulators that increase osteoclast apoptosis[34]. Notably, the endocrine and paracrine roles of adipokines in bone metabolism are contradictory. The results from *in vitro* and *in vivo* studies are sometimes contradictory, and no direct correlation between increased levels of adipokines and supported bone metabolism could be clearly stated. Several factors could be involved, such as dosage, the timing of adipokine release and their effect in mediating inflammation[35]. It appears more likely that WAT and bone metabolism crosstalk is contextual. The presence of adipokine receptors on BMSCs and osteoblasts demonstrates that anabolic bone metabolism has a direct interrelation with WAT adipocytes. The WAT-bone catabolic interrelation is exerted through distinct pathways that regulate osteoclast formation or apoptosis.

WAT contains an important fraction of immune cells. Local AT-resident Mcfs (MATs) in normal/lean individuals display an anti-inflammatory phenotype described as the M2 (alternatively activated) phenotype, which supports WAT expansion during adaptation to a high-fat diet. Prolonged WAT inflammation, however, leads to fibrosis and ECM stiffness, hindering adipocyte expansion and lipid storage[36]. WAT expansion during obesity is associated with increased levels of macrophage chemoattractant-1, which determines the accumulation of high levels of M1 (inflammatory) MATs that can induce insulin resistance[37]. Other local immune cells, regulatory T cells (Tregs), which are more abundant in lean but not obese WAT, have been shown to promote the M2 MAT phenotype, and their increase in obese WAT can improve insulin sensitivity[38].

Mcfs are present during the inflammatory phase of fracture healing in humans and animals. Compared to Mcf derived from blood monocytes, tissue-resident cells seem to have a more important role in fracture healing. Bone marrow and periosteal M1 phenotype Mcf-released cytokines [interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)] are present at the fracture site during the first days after injury in animal models and humans. M1 phenotype persistence during callus formation delays or compromises healing. Bone-specific Mcfs (osteomacs) have been involved in bone healing and remodelling; however, other Mcf tissues might also contribute[39]. Tissue-resident M1 and M2 MCfs are involved in *de novo* angiogenesis within the granulation tissue during fracture healing, as well as stem cell/progenitor cell recruitment to the fracture site and their differentiation. Oncostatin M, a cytokine of the IL-5 family produced by M1 Mcfs, was shown to induce osteoblast differentiation and matrix mineralization from human mesenchymal stem cells while inhibiting adipogenesis *in vitro*[40]. Furthermore, the number but most of all the Mcf phenotype required for fracture healing might be dependent on the type of callus formation (enchondral *vs* endosteal) and the type of fracture fixation. More detailed *in vivo* studies are required to decipher the origin of Mcf involved in fracture healing; however, these cells apparently need to switch phenotype upon environmental stimuli during the time course of bone healing. MAT involvement in bone remodeling and fracture healing needs further investigation. Delayed fracture healing during obesity and metabolic syndrome might be[41], at least in part, explained by the presence of M1MAT, which prolongs the inflammatory stage and prevents callus maturation.

BAT

The typical BAT is located between the shoulder blades in smaller mammals. In newborn humans, the interscapular "BAT organ" contains adipocytes that are multilocular dispersed as lipid droplets within the cytoplasm and contain numerous mitochondria that express uncoupling protein-1 (UCP-1). Their main function is to metabolize FAs for thermogenesis, protecting the body from cold exposure through non-shivering thermogenesis[42]. Two forms of BAT are currently recognized to exist in humans:

Constitutional BAT (cBAT) formed during embryonic development and beige or brite (brown-in-white) BAT. The former is recruited postnatally from WAT and has been denominated recruited BAT (rBAT). cBAT of developmental origin seems to be mesoderm closer to skeletal muscle rather than to AT, while rBAT can be formed after birth by transdifferentiating mature white to brown adipocytes or by differentiation from BAP[43]. Previously considered to be represented in humans only in newborns, recent positron emission tomography/computed tomography (PET/CT) studies based on imagistic detection of UCP-1-positive adipocytes have identified functional BAT in adults. BAT seems to be more frequent in women than in men and is inversely correlated with BMI, especially for elderly subjects[44].

The main function of BAT is thermal regulation. Recently, several studies in mice revealed the role of BAT as a negative regulator of obesity since UCP-1 depletion in mice induced increased cold sensitivity and obesity[45]. Apart from connections with energy metabolism and thermal regulation, recent studies correlated BAT with bone anabolism[46]. BAT detected around the neck in the supraclavicular region and paravertebrae using functional PET-CT correlated positively with BMD in women[47] but not in men[48]. This possible sex dependence of the positive effect of BAT on bone was not confirmed in a cross-sectional study correlating BAT volume with femoral cortical bone area and cross-sectional area in children and teenagers independent of sex[49]. The transcriptional regulator and tumor suppressor retinoblastoma-associated protein (pRb) have been identified as a possible connection between bone and BAT and bone turnover. pRB functions as the switch mechanism that directs mesenchymal progenitors to the osteoblastic lineage, while deletion of pRb in the respective precursors increased the amount of BAT in mouse models[50]. BMP overexpression in soft tissues after trauma seeks to recruit brown adipocytes and induce hypoxia-mediated chondrogenic differentiation of local progenitors. Subsequent ossification of chondrogenic nodules determines the formation of posttraumatic heterotopic ossification [51].

BAT AND BONE METABOLISM

Consistent research is directed to finding methods of transforming white adipocytes into brown or "beige" phenotypes for the treatment of obesity and associated metabolic disorders. Overexpression of forkhead transcription factor C2 (FOXC2) in mouse WAT cells induced a BAT-like phenotype[52]. Genetically modified mice that overexpress FOXC2 were found to not only be protected against diet-induced obesity and insulin resistance but also have increased trabecular bone mass and bone turnover [53]. BAT-bone metabolism could be correlated through secreted paracrine factors. AT and BMAT overexpressing FOXC2 displayed increased gene expression of endocrine factors adiponectin, insulin growth factor receptor 3 (IGFR2) and IGF1, as well as paracrine factors BMP4, wingless-type MMTV integration site family member 10B and angiopoietin 2. Human endolymphatic sac epithelial factors were shown to exert a pro-osteoblastic effect *in vitro* and could represent the modality of BAT-bone communication and bone anabolic support[46].

BMAT - A UNIQUE TYPE OF AT

Bone marrow contains a fraction of AT that fluctuates during development, growth, ageing and pathological conditions. While this fact is common knowledge, the origin, role and functions of BMAT remain largely unknown. More closely resembling WAT, which shares several microstructural commonalities[54], BMAT has a particular molecular make-up that distinguishes it from WAT and BAT[55]. The unicity of BMAT seems to be related to not only its particular anatomic location and spatial constraints but also its involvement in body functioning as a whole[56]. BMAT-released adipokines, inflammatory cytokines and other possible bioactive molecules are thought to exert systemic regulatory effects. Recent years have witnessed a surge in experimental investigations that challenge the passive role of BMAT as a simple space filler within the bone marrow microenvironment. The onset and progression of postmenopausal osteoporosis in the context of oestrogen depletion[57], glucose homeostasis, energy metabolism[58], or adipocyte-osteoblast balance have been recently linked with BMAT reactivity[59]. These investigations point towards BMAT involvement in structural changes occurring within the skeleton with age during physiological and pathological situations and as a key player in the maintenance of body energetic expenditure.

STRUCTURE AND COMPOSITION OF BMAT

In healthy adults, BMAT represents approximately 10% of the total body AT. While the presence of adipocytes within the complexity of the bone marrow tissue environment was detected a century ago, only recently was their role as a local and systemic regulator explored[60]. The availability of methods for *in vivo* quantification and the use of reporter transgenic mice combined with experimental induction

of BMAT expansion have enabled recent insights into its function. The correlation of BMAT expansion with metabolic diseases, such as obesity, metabolic syndrome, diabetes and anorexia nervosa, is sought to point towards a role in systemic metabolic balance. Unlike any other form of AT, BMAT has physical vicinity at a cellular level with bone tissue. BMAT expansion is associated with decreased bone mass and osteoporosis experimentally, as well as in epidemiological studies[61]. Multiple factors are involved in this correlation, such as bone marrow mesenchymal stem cell adipogenic *vs* osteoblastic conversion, adipokine release or inflammation. BMAT is also involved in normal and pathological haematopoiesis through adipocyte cellular interaction with haematopoietic progenitors and local adipokine release[62]. BMAT adipocytes are responsive to producing and sustaining a local inflammatory environment that impacts *de novo* bone formation and favors malignant conversion of haematopoietic lineages or tumor metastasis to bone[63].

Considered to originate from bone marrow mesenchymal progenitors, BMAT adipocytes are unilocular similar to WAT and can be found within the bone marrow cavity of bones. In young individuals in humans and mice, bone marrow has a red appearance and contains predominately haematopoietic and osteoblast progenitors, as well as erythroid cells. Macroscopically, bone marrow becomes yellow with a fatty structure upon BMAT development. Using magnetic resonance imaging, in human subjects, progression of red to yellow marrow was observed in the long bones (the femur) first in the diaphysis (ages 1-10 years) and then in the distal metaphysis (ages 10-20 years), with an adult pattern seen by age 24 years[64]. BMAT first develops in the distal appendicular skeleton (femur, tibia) compared to the proximal and caudal vertebrae (tail bones) compared to the proximal (thoracic) vertebrae. In rats, differences were attributed to cold exposure, as well as strong erythropoietin stimuli, since retaining warm temperature, as well as induced haemolysis, prevented bone marrow "yellowing" in pre-weanling but not in mature animals. This led to the conclusion that BMAT once formed is a stable tissue[65]. Two developmental and regional distinct BMAT subtypes have been identified. The distal localized, first to develop, was denominated the constitutive (cBMAT), while the proximal placed (within proximal limbs, thoracic vertebrae, hips, ribs) later occurring and more scattered was denominated the regulated (rBMAT). cBMAT was found to contain predominately unsaturated lipids, while rBMAT contains saturated fats. It has been proposed that rBMAT can mature into the more stable cBMAT phenotype under certain conditions[57].

ROLE OF BMAT IN BONE METABOLISM AND FRACTURE HEALING

BMAT adipocytes are a major participant in the BM niche alongside BMSCs and hematopoietic stem cells. Their physical presence, as well as endocrine and paracrine function, impacts osteoblast and osteoclast differentiation and functioning[66]. Several mechanisms for BMAT adipocyte involvement in the maintenance of bone anabolic-catabolic balance have been proposed.

Since osteoblasts and adipocytes share a common precursor, the most important factor in regulating bone formation is intrinsic BMSC "fate decision". Lineage determination is controlled on one side by signalling pathways that promote expansion of one lineage *vs* another and on the other side by suppression of pathways promoting the competitive lineage. Bone formation occurs by inducing osteogenic key regulators runt-related transcription factor 2 (RUNX2) and osterix in MSCs while inhibiting adipogenic PPAR-γ and CCAAT/enhancer-binding protein α *via* a Wingless-type MMTV integration site family (Wnt) mechanism[67]. Conversely, adipogenesis requires concomitant induction of key adipogenic pathways and inhibition of osteogenic Wnt and Notch[68]. In BMSCs, intracellular accumulation of proteins that induce adipogenesis, such as transducing-like enhancer of split 3, increases the expression of PPAR-γ and suppresses Wnt-induced β catenin accumulation and RUNX by a histone deacetylase mechanism[69]. Increased BMSC adipogenic conversion and reduced osteoblast formation are considered the main culprits for compromised bone anabolism and BMAT accumulation [70]. It is currently accepted that an increase in BMAT during ageing, osteoporosis, and T2D is associated with decreased bone quality and quantity (osteoporosis, osteopenia). However, this inverse correlation is not confirmed by all clinical situations. Epidemiological studies confirm increased BMAT in osteoporotic patients compared to age-matched controls in children, young adults and elderly individuals[71,72]. Increased BMAT was found to correlate with increased BMD in obese and T2D patients[73]. Furthermore, decreased BMD and increased BMAT content in anorexia nervosa are associated with decreased BMI in anorexia nervosa patients[74]. These findings suggest that BMD might not be the ultimate predictor of bone quality and that BMSC adipogenic and osteoblast conversion might not be mutually exclusive[75]. Lineage tracking of adult adipocyte BMAT is warranted to elucidate their origin, as well as potential competition with osteoblast differentiation and maturation.

The BMAT-bone relationship does not resume cell fate decisions. MAT-released adipocytes (especially leptin and adiponectin), inflammatory cytokines (which include TNF-α and the IL family) and mRNA-containing extracellular vesicles (EVs) form a complex signalling network involved in regulating osteogenesis[76]. It is worth mentioning that studies on AT-released factors and their influence on bone metabolism largely involve WAT adipocytes. Few studies specifically address the molecular mechanisms of BMAT adipocyte-secreted signalling molecules and their role in bone

metabolism.

No direct evidence exists regarding the association between BMAT and fracture risk and fracture healing. Indirect observation is provided by studies on fracture healing in obese experimental models of human subjects that could have increased BMAT. Experimental studies on obese mice reported an increased incidence of delayed union associated with increased callus adiposity in obese T2D mice[77, 78]. A meta-analysis of eight epidemiologic studies including 39938 participants concluded that metabolic syndrome has no explicit effect on bone fractures[79]. In another study, obesity was not associated with an increased incidence of nonunion after ankle fractures[80], while yet another study reports a greater risk of complications in obese patients[81]. Multiple confounders, such as the association of alcohol consumption, T2D, and quality of fixation, can explain these contradictory results. Another possible indirect indication comes from the studies reporting increased fracture healing in patients with long bone fractures fixed with reamed intramedullary rods *vs* non-reamed patients. This finding can be explained by the stability of fixation, preservation of fracture haematoma that favors formation of periosteal callus or activation of MSC recruitment[82], rather than by mere removal of BMAT.

AT IN BONE REGENERATIVE MEDICINE

Regenerative medicine (RM) aims to completely restore functionality and anatomy in degenerating or ageing tissues or to replace tissues and organs lost to trauma, infection, tumor removal or congenitally absent[83]. RM makes use of cells, especially stem cells, bioactive molecules and supportive/functional ECM equivalents, to induce regeneration or engineer implantable bioequivalent structures. Recent decades have witnessed a surge in regenerative interventions for improving bone health, aiming to increase bone quality and prevent or treat failures in fracture healing. Several cell types of use for RM purposes can be obtained from AT, and adipose-derived mesenchymal stem cells (ADSCs) and adipose-derived pluripotent stem cells will be briefly described in the following subchapters.

ADSCS

AT is considered a convenient source of mesenchymal stem cells because of its ease of procurement and abundance of colony-forming units. Compared to adult bone marrow, the frequency of ADSCs obtained per tissue unit can be up to 500-fold higher[84]. AT can be obtained by minimally invasive procedures (subcutaneous lipectomy) or as a byproduct of cosmetic liposuction procedures. ADSCs were first obtained from subcutaneous WAT lipoaspirate by enzymatic digestion and selection of plastic adherent cell populations[85]. Enzymatic digestion of lipoaspirate or WAT fragments obtained by lipectomy or mechanical cell extraction from the same sources derives the stromal vascular fraction (SVF). SVF is a cell mixture that contains preadipocytes, fibroblasts, vascular cells, blood cells and Mcfs that can be readily used for regenerative purposes. ADSCs are obtained from the SVF by further cultivation and selection of mesenchymal progenitors based on their adherence to tissue culture. The anatomic location of harvest (such as abdominal, brachial, inguinal) position (superficial subcutaneous *vs* deep hypodermic), age and sex of the donor influence the number of mononuclear cells extracted and the number of ADSCs obtained from subcutaneous WAT[86]. ADSCs meet the criteria established by the International Society for Cell Therapy for defining mesenchymal progenitors (plastic adherence, trilineage mesenchymal differentiation and surface marker phenotype)[87]. It has been reported that the SVF contains four different mesenchymal cells or progenitors or that the putative ADSCs are CD31-, CD34+/-, CD45-, CD90+, CD105-, CD117- and CD146-, the others being pericytes (CD146+/CD31-/CD34-), mature endothelial cells (CD31+/CD34-), progenitor endothelial cells (CD31+CD34+), and preadipocytes as CD31-/CD34+ cells[88]. ADSCs were reported to differentiate under controlled conditions *in vitro* to mesenchymal lineages (adipocytes, chondrocytes, osteoblasts and cardiomyocytes [89] and skeletal muscle[90]). Ectodermal (neurons, glia and Schwan cells) and endodermal (hepatocytes and pancreatic beta islet cells) ADSC conversion has been obtained[91]. A subset of ADSCs was shown to express markers of pluripotency (Sox2, Nanog, and OCT4) and to differentiate into mesodermal and extramesodermal lineages, especially when cultured in three-dimensional suspension culture[92]. An important feature of putative ADSCs is their growth factor and immunomodulatory cytokine release. ADSCs were found to express multiple growth factors, of which basic fibroblast growth factor (bFGF), VEGF, insulin-like growth factor 1, HGFs, and transforming growth factor (TGF)-β1 but as well β-nerve growth factor, stromal cell-derived factor-1α and growth factor receptors. Mass spectrometry analysis of the ADSC secretome revealed that ADSCs express 342 proteins under normoxic conditions. These proteins were found to be related to angiogenesis and blood vessel expansion, ECM formation, cell adhesion/migration, cell survival/death, and immune regulation with little variation after hypoxic preconditioning[93]. Importantly, the ADSC secretome varies upon stimulation. bFGF or epidermal growth factor (EGF) preconditioning significantly increases ADSC release of HGF, a cytokine involved in haematopoiesis, vasculogenesis, and mammary epithelial duct formation[94]. Neural growth factor

preconditioning increased the axonal growth capability of a conditioned medium from ADSCs[95]. It has been proposed that preconditioning ADSCs using low oxygen content, generation of reactive oxygen species (ROS) and activation of platelet-derived growth factor (PDGF) receptor signalling can increase the regenerative proprieties of cultivated ADSCs by mimicking the *in vivo* regenerating niche [96].

Inflammatory cytokine release varies upon ADSC stimulation. Exposure to lipopolysaccharides induced the release of haematopoietic (granulocyte/monocyte, granulocyte, and macrophage colony-stimulating factors, IL-7) and proinflammatory mediators (IL-6, IL-8, and IL-11, TNF-α)[97]. Under normal culture conditions, conditioned medium from ADSCs reduced the production of TNF-α, NO and prostaglandin E2, and the activation of nuclear factor-kappaB in blood-derived monocytes decreased their degranulation, phagocytic activity and migratory ability. Notably, using next-generation sequencing, cultivated ADSCs were found to have a more homogenous immunomodulatory gene expression profile than SVF in the natural state and upon TNF-α stimulation[98]. Trophic and immunomodulatory factors released by cultivated ADSCs are strongly influenced by a large variety of factors, such as WAT origin, donor age and health status, cell culture and preconditioning[99-101] (for a summary, see Table 1). While this influence opens large possibilities in manipulating cell therapeutic qualities, it calls for thorough characterization when an ADSC-based product is envisaged.

Given their phenotypic characteristics, ADSCs are intensively sought for their differentiation and tissue trophic and immunomodulatory potential. ADSCs can be used as building blocks for *de novo* bioengineered organs and are currently tested for the generation of musculoskeletal tissues[102,103]. Cell therapy using ADSCs has proven useful in preclinical settings for immunomodulation in autoimmune diseases (such as inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis) [104]. With the recent coronavirus disease 2019 pandemic, ADSCs have been tested in emergency clinical trials for the prevention of severe "cytokine storm" and the installation of acute respiratory distress syndrome, septic shock, and/or multiple organ failure[105].

ADSCs have been intensively tested *in vitro* and in preclinical studies for their direct contribution by differentiation to the osteoblastic lineage, for their supportive effect in promoting osteogenesis and for accelerating fracture healing. Deriving from these distinct roles in RM, bone tissue engineering using ADSCs as cell sources and cell therapy for the treatment of problematic bone healing, bone pathology and systemic osteoporosis are possible therapeutic scenarios.

DIRECT EFFECT OF ADSC IN REGENERATION - OSTEOBLASTIC DIFFERENTIATION AND TISSUE-ENGINEERED BONE STRUCTURES

Numerous reports exist regarding the *in vitro* osteogenic potential of ADSCs under defined differentiation media, and osteogenic conversion is assessed based on specific gene expression (OC, core-binding factor subunit alpha-1, also known as RUNX2, AP, osteonectin, osteopontin, BMP-2, ALP activity and mineralized ECM deposition)[106]. Mechanical stimulation by dynamic compression or magnetic nanoparticle-induced remote actuation has also been reported to increase *in vitro* osteogenesis [107,108]. To assess ADSC osteogenic potential *in vivo*, several methods have been validated starting from ectopic bone formation in small animal models (rat, mice) after intramuscular delivery of osteogenic-induced ADSCs[109]. More complex models consist of healing experimentally induced calvarial bone defects in rodents or long bone fractures[110,111]. Generally, *in vivo* testing of ADSC osteogenic potential requires the use of a supportive structure for cell implantation. This strategy realizes a tissue-engineered implantable structure with variable degrees of complexity and potential for clinical translation. The classical "tissue engineering triad" is based on the use of scaffolds, cells and bioactive molecules to generate implantable bioequivalent tissues or organs. For bone engineering, the biomaterial needs to fulfil the general requirements for a scaffold structure (biocompatibility, biodegradability, porosity and interconnectivity of the pores, not to generate inflammatory response *in vivo*). In addition, this material needs to be osteoconductive (to allow bone mineral and collagen deposition) and osteoinductive (to favor osteogenic differentiation). Three main types of biomaterials have been used for scaffolds in bone tissue engineering: Ceramics (such as tricalcium phosphate, hydroxyapatite and combinations) and synthetic polymers [such as polylactic acid, polyglycolic acid (PGA), and poly-DL-lactic-co-glycolic acid]. Natural polymers, such as collagen, hyaluronic acid, chitosan, fibrin, and elastin, have been used alone or in combination with synthetic polymers or with ceramics[112]. Alongside the osteoconductive and osteoinductive properties, biomaterial osteointegration is crucial for the stability of the engineered graft. Osteo integration is dependent on blood vessel colonization from the surrounding host tissue that allows for nutrient supply, waste removal and erasing of implant host interfaces that impede mechanical stability. Especially in the case of larger constructs, the biomaterial needs to be angioconductive and permissive to vascular in growth. Angioinduction, the ability of a biomaterial to actively induce and sustain the formation of new vessels, is another determinant of osteointegration accounting for adequate vascular supply and long-term stability of the engineered bone[113].

Table 1 Factors that influence adipose-derived mesenchymal stem cells secretome content and release[99-101]

Donor	Tissue	Culture conditions	Cell manipulation
Species	Type (sWAT, vWAT)	2D *vs* 3D (spheroid culture, cell sheets), suspension culture	Preconditioning (IFN-γ, TNF-α, LPS)
Age	Anatomic location (abdominal, brachial, mammary)	Hypoxia	Differentiation (osteogenic, adipogenic)
BMD (obesity)	Method of procurement (lipectomy, liposuction)	Plating density, co-culture	Physical factors (electromagnetic fields, pulsed electromagnetic fields)
Disease (T2D, metabolic syndrome, lipodystrophy)	Methods of tissue processing, enzymatic digestion, mechanical trituration	Media formulation (serum containing versus serum free, growth factor addition)	

T2D: Type 2 diabetes mellitus; sWAT: Subcutaneous white adipose tissue; vWAT: Visceral white adipose tissue; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor alpha; LPS: Lipopolysaccharides; BMD: Bone mineral density.

Advanced nanostructured materials with remarkable properties are promising for revolutionizing the field of bone engineering. Graphene, with its high surface area, high mechanical strength, and high functionalization potential, can induce ADSC differentiation even in the absence of osteogenic media. The feasibility of generating mechanically stable graphene-based implantable bone grafts and the *in vivo* osteoinductive capabilities of these implants need to be further tested[114].

Bioactive molecules largely employed for bone tissue engineering are osteoinductive growth factors from the BMP family. BMP-2 was clinically approved by the Food and Drug Administration for spine fusion, and BMP-7 was given a device exemption for the treatment of nonunions. As a result, many studies began investigating BMP as a modality to enhance ADSC-based osteogenesis *in vivo*, envisaging smoother clinical translation. However, since activation of the BMP pathway in ADSCs induces osteogenesis and adipogenesis, the use of BMP alone cannot always account for the desired fate decision. To shift the balance towards osteogenesis, switches such as the Wnt and extracellular signal-regulated kinase pathways and the ratio between BMP receptors bone morphogenetic protein type IA receptor (BMPR-IA)/BMPR-IB are at play. Controlling the sequential cascade of growth factor availability *in vivo* can prove to be technically challenging. Several methods, such as controlled release, scaffold-mediated release, gene transfer technologies or stimulation of endogenous BMP activation, have been proposed[115].

Several growth factors relevant for osteogenesis, such as b-FGF or FGF-2, IGF-1, PDGF-BB, and VEGF, are contained in platelet-rich plasma (PRP), a blood-derived biologic that is easy to procure from autologous sources. PRP incorporated within composite hydrogel-ceramic scaffolds yielded increased osteogenic ADSC conversion in a rabbit calvarial model compared to non-PRP-treated implants[116]. Different strategies of PRP coating of synthetic electrospun scaffolds appear promising, awaiting further tests for *in vivo* validation of the procedure[117]. Alternatively, overexpression of different transcription factors in ADSCs (RUNX2, VEGF, sonic hedgehog, and LIM mineralization protein) was shown to increase osteogenic differentiation and could prove an efficient strategy for inducing bone formation *in vivo*[118].

Another strategy of GF delivery could be *in vitro* cell preconditioning with osteoinductive molecules. FGF2-pretreated human ADSCs showed enhanced *in vivo* osteogenic potential in an ectopic bone model and increased osteoid formation in a dose-dependent manner[119]. Exosomes are EVs of endosomal origin, ranging from 50-200 nm in diameter, that function as intracellular communication tools. MSCs, especially ADSC EVs, contain cell-specific proteins (cytoskeletal proteins, transmembrane proteins, and heat shock proteins), nucleic acids [DNA, mRNA, micro RNA (miRNA), long and short noncoding RNA], lipids, and enzymes. EVs are recognized as bioactive cargoes with importance for cell recruitment, migration, proliferation, and *de novo* vascularization and have an important impact on tissue regeneration[120]. ADSC-derived EVs have been investigated as potential tools for inducing osteogenic differentiation. The PLDA/PGA matrix slowly released EVs from osteogenic-induced ADSCs and was shown to promote osteogenesis of BMSCs *in vitro*. Furthermore, cell-free PLDA/PGA-EV increased osteogenesis in a mouse calvarial model compared to the PLDA/PGA matrix only[121]. EVs from osteogenic-induced ADSCs could promote osteogenesis in undifferentiated ADSCs. Remarkably, ADSCs could incorporate EVs faster than BMSCs (6 h compared to 48 h), which could be of importance for therapeutic applications. Even though the study was not validated *in vivo*, the authors performed microarray gene expression and bioinformatics analyses, revealing that the differentially expressed exosomal miRNAs from osteogenic-induced ADSCs compared to undifferentiated ADSCs are involved in the osteogenetic process (the MAPK, Wnt, and TGF-β signalling pathways). The expression levels of miR-130a-3p, which blocks SIRT7, an antagonist of the Wnt pathway, were found to be significantly higher in EVs from osteogenic ADSCs. MiR-130a-p ultimately upregulates the Wnt pathway, possibly acting as the molecular mechanism of increased ADSC osteogenic induction by EVs

[122].

Given their increasingly recognized role in modulating osteogenesis, miRNAs or inhibitors have been tested for inducing ADSC differentiation. Scaffold-mediated release to ADSCs or virus-transfected miR-148b, miR-26a, miR-135, or miR-130a-3p was found to increase bone formation *in vitro* and *in vivo*[123, 124]. Other miRNAs, such as miR146a, miR-17, miR-23a, and miR-31, were found to inhibit BMP2-induced osteogenesis, suppressing downstream factors in BMP-2-induced osteogenesis (such as RUNX2, Osterix, and SMAD1/4). Antisense inhibition of these miRNAs in ADSCs seeded on a β-tricalcium phosphate scaffold was found to increase bone volume and BMD and to decrease scaffold residue persistence in critical size bone defects in rats[125].

Mechanical stimulation is crucial for obtaining bioengineered structures, especially in the case of musculoskeletal components. Functional tissue engineering is set to obtain robust bioequivalents that readily restore the morphology and load-bearing and motion capabilities of bone. A variety of mechanical loading procedures that apply cyclic hydrostatic pressure or tensile strain in dynamic culture conditions have been used to increase ADSC osteogenesis[126]. Magnetomechanical stimulation using magnetic nanoparticles internalized by ADSCs and magnetic field exposure during the first phases of osteogenesis has been reported as a modality to deliver remote controlled and device-free mechanical stimulation[108] (Figure 1).

A consistent number of preclinical studies have reported the use of various combinations of supportive structures, bioactive molecules and/or functional loading for testing ADSC osteogenic capability *in vivo*. Reports about the successful use of ADSC-based tissue-engineered bone are abundant in the literature[56]. Despite these encouraging results, translation to clinical settings has proven more difficult. The first report of clinical use was made in 2004 and involved ADSC use in a paediatric patient. A large calvarial posttraumatic bone defect was treated with autologous ADSCs and iliac crest cancellous bone autografts, fibrin glue and resorbable macroporous sheets[127]. In the years to follow, several case reports emerged regarding the use of autologous ADSCs and clinically approved bone substitutes with or without BMPs for grafting of craniofacial bone defects (mandibular and maxillary bone)[128-130]. The combination of autologous ADSCs expanded in good manufacturing practice facilities and ceramic bone substitutes resulted in uneventful healing of bone defects. Cranioplasty of large calvarial defects using autologous ADSCs and β-transmission control protocol was reported as a useful method to replace massive bone loss[131]. Remarkably, all clinical reports regarding ADSC use involve the reconstruction of craniofacial bone defects. To our knowledge, recent years have not added to the reported clinical studies in this field. A list of current clinical trials registered on clinicaltrials.gov is available in Table 2.

THE SUPPORTIVE ROLE OF ADSC - CELL THERAPY FOR BONE DISEASES AND FOR AUGMENTING FRACTURE HEALING

The trophic role of ADSCs in tissue has been investigated for the treatment of metabolic bone diseases, such as osteoporosis. As a multifactorial disorder, osteoporosis has external and intrinsic determinants and is commonly associated with postmenopausal hormone depletion, ageing or long-term use of corticosteroid medication[132]. Local or systemic delivery of ADSC suspensions as cell therapy is sought to modulate bone resorption, increase bone formation and enhance BMD. The procedure relies less on the capability of infused cells to differentiate into osteoblastic lineages but rather on cytokine and growth factor release. This paracrine activity is expected to increase osteoprogenitor cell recruitment, proliferation, differentiation, ECM formation and mineralization[133]. Several preclinical studies report on the efficiency of autologous locally delivered ADSCs in improving bone strength in ovariectomized rats or in senescent mice[134,135]. Systemic human ADSC delivery in ovariectomized nude mice was as effective as oestrogen therapy in protecting trabecular bone loss, without evidence of ADSC engraftment[136].

Osteonecrosis of the femoral head (ONFH) is considered to be produced by apoptosis of mature osteocytes mainly due to impaired blood supply. ONFH affects a younger population, leading to collapse of the femoral head, a situation that requires total joint replacement. Unlike other forms of cell therapy, in ONFH, the use of stem cells started in clinics with the use of bone marrow aspirate concentrate as a modality to deliver progenitor cells locally after core decompression[137]. Most studies regarding the use of cultured MSCs for ONFH involve BMSCs; however, coculture with ADSCs was reported to have a synergistic effect mainly due to ADSC angiogenic potential[138]. Stem cells are commonly delivered within a supportive structure, such as fibrin gel or bone substitute for retaining the cells, as well as a modality to support or to prevent the collapse of the femoral head. Implanted cell contribution is probably rather paracrine because the local environment is not favorable for cell survival and differentiation after transplantation. ADSCs have been tested as a modality to locally deliver angiogenic factors. VEGF-transfected ADSCs in coculture with BMSCs were effective in inducing osteogenesis and angiogenesis *in vitro* and *in vivo*; however, this role needs to be further tested in ONFH animal models[139].

Table 2 Clinical trials using adipose-derived mesenchymal stem cells for bone regeneration registered on clinicaltrials.gov (June 2021)

Trial registration	Ref.	Study design	Group/cohort	Intervention	Location	Status
NCT01218945	Calcagni [168]	Observational/prospective	Overweight, non-metabolic disease, 17-80 yr	To pre-engineer large synthetic bone grafts and study the vascularization process *in vivo*	University of Zurich, Switzerland	Completed December 2012
NCT01532076	Saxer and Jakob [169]	Allocation: Randomized. Intervention model: Parallel assignment. Masking: Single (outcomes assessor). Primary purpose: Treatment	Patients with osteoporotic fractures, age 18-70 yr	Cellularized composite graft augmentation liposuction, cell isolation, embedding of SVF cells in fibrin gel, wrapping around hydroxyapatite granules compared to acellular grafts	University Hospital, Basel, Switzerland	Terminated/slow recruitment rate/last update 17 September 2014
NCT01643655	Yoon [170]	Allocation: N/A. Intervention model: Single group assignment. Masking: None (open label). Primary purpose: Treatment	Avascular necrosis femoral head Steinberg I-III/pre-collapse, age 17-70 yr	Autologous adipose tissue-derived MSCs transplantation into the femoral head/infusion of autologous adipose-derived mesenchymal stem cells. Dose: 1 × 10^8 cells/3 mL	R-Bio SMG-SNU Boramae Medical Center	Recruitment completed/last update 31 August 2017
NCT02140528	Gourabi *et al*[171]	Allocation: Randomized. Intervention model: Parallel assignment. Masking: Double (participant, investigator). Primary purpose: Treatment	Closed shaft tibial fracture, age 18-65 yr	Injection of adipose-derived mesenchymal stem cell in the site of tibia fracture. Other name: Stem cell transplantation compared to Placebo	Royan Institute Tehran, Iran	Completed/last update 27 April 2017
NCT03269409	Sierra [172]	Interventional, allocation: Randomized. Intervention model: Parallel assignment. Masking: Quadruple (participant, care provider, investigator, outcomes assessor). Primary purpose: Treatment	Patients with ONFH pre-collapse, non-posttraumatic, 22-70 yr of age	Adipose-derived regenerative cells harvested through autologous liposuction processed using the Celution 800/GP System (Cytori Therapeutics) transplanted into the femoral head after standard of care hip decompression compared to standard decompression and Ringer solution	Mayo Clinic, United States	Suspended (updating study protocol, consent form and study SOP protocol) March 2021
NCT02307435	Dilogo [173]	Allocation: N/A. Intervention model: Single group assignment. Masking: None (open label). Primary purpose: Treatment	Fracture nonunion metaphyseal fibrous defect, age 19-30 yr	Experimental: Implantation group implantation group will receive ADSC/UCMASC/BMSCs and HA-$CaSO_4$. Intervention: Biological: MSC	University of Indonesia, Jakarta	Unknown/last update 4 December 2014
NCT03678831	Pasquier [174]	Observational, case-control prospective	Arthritic post-menopausal patients with knee prosthetic replacement	Adipocyte isolation from distal femoral epiphysis and subcutaneous adipose tissue at the surgery site; classical piece removal during prosthetic replacement of the knee	University Hospital, Lille, France	Recruiting/April 2021
NCT04377880	Fodzo [175]	Observational study	Osteoporotic patients undergoing total joint arthroplasty	Osteoblastic response to medullary adipocytes of commercial origin analysed by gene expression and correlation with clinical data regarding osteoporosis and microtomography	University Hospital, Lille	May 2021

ADSC: Adipose-derived mesenchymal stem cells; UCMASC: Umbilical cord mesenchymal stem cell; BMSC: Bone marrow stem cell; MSC: Marrow stem cell; ONFH: Osteonecrosis of the femoral head; SOP: Standard operating procedures; SVF: Stromal vascular fraction.

Delayed or impaired fracture healing can complicate up to 10% of total fracture cases[140]. Local risk factors can affect the quality and speed of bone healing, such as the severity of bone and soft tissue injury and the coexistence of multiple fractures or other associated trauma. Systemic factors, such as diabetes, obesity, malnutrition, smoking, and advanced age, are also known to represent a high risk for bone healing. ADSCs have been tested as a method for increasing the quality and decreasing the time of bone healing in animal models. Human ADSCs and their conditioned medium embedded in human blood plasma hydrogel were shown to increase fracture healing in surgically induced rat jaw fracture, demonstrating their paracrine effect in promoting bone union[141]. Local ADSC injection in healthy and diabetic rat femoral nonunions induced significant bone healing, as assessed by histology, compared to nontreated groups independent of RANK, RANKL, or OPG gene expression[142]. A combination of human ADSCs, cancellous bone grafts and chitosan gel consistently improved healing of the surgically induced nonunion of the femoral bone in rats, as confirmed by biomechanical and histological studies. ADSC presence was correlated with increased expression of VEGF and BMPs in the treated groups [143]. Autologous ADSCs delivered by local injection in atrophic nonunions in rat tibia resulted in significantly increased callus and solid bone union[144]. The report represents proof of concept of ADSC

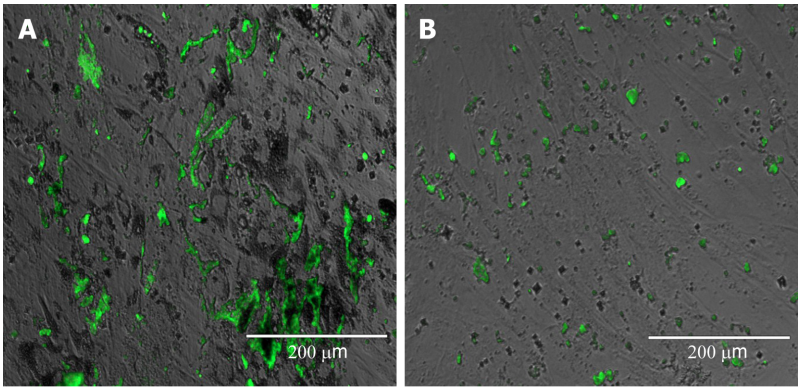

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Figure 1 Adipose-derived mesenchymal stem cell osteogenesis under magnetomechanical stimulation. A: Osteogenesis of adipose-derived mesenchymal stem cells (ADSCs) loaded with micronutrient powders (MNPs) exposed to alternating MFs; B: Osteogenesis of ADSCs without MNPs exposed to MFs assessed with OsteoImage® Lonza showing green fluorescence for the deposited calcified extracellular matrix.

regenerative capabilities even in this difficult-to-treat variety of impaired fracture healing. The clinical use of ADSCs as a cell therapy for enhancing fracture healing has not yet been reported.

WAT-DERIVED PLURIPOTENT CELL POPULATIONS: MULTILINEAGE DIFFERENTIATING STRESS-ENDURING CELLS AND DEDIFFERENTIATED FAT

WAT is the source of two cell populations with tripoblastic differentiation potential and expression surface markers of pluripotency. Multilineage differentiating stress-enduring (MUSE) cells were initially obtained from dermal fibroblasts and BMSCs as stress-resistant populations[145]. WAT was soon identified as a plentiful source of MUSE cells that could be obtained by means of positive immune separation for mesenchymal surface antigen CD105 and pluripotency marker stage-specific embryonic antigen 1 (SSEA-1)[146]. A remarkable characteristic of these cells is their ability to grow in adherent and suspension culture conditions. When MUSE cells are cultured in a single-cell suspension, they form so-called "M clusters" with morphological resemblance to ESC or induced pluripotent stem cell (IPSC) embryoid bodies formed from embryonic stem cells (ESCs) or IPSCs. Since MUSE cells do not generate tumors after *in vivo* injection into severe combined immunodeficient (SCID) mice, they are considered safer than ESCs or IPSCs. MUSE cells are a small percentage of tissue-derived MSCs and are considered to be responsive to the regenerative potential of these populations. Their ability to migrate to damaged tissue and to spontaneously differentiate into cells that pertain to damaged tissue is regarded as having important potential in RM since unlikely ESC or IPSC preinduction to the respective lineage is not necessary[147]. MUSE cells have been tested in animal models for cardiovascular rescue (myocardial infarction) ischaemic stroke, lung injuries, kidney diseases and skin repair[148]. Their use in bone regeneration has not yet been tested; however, good results obtained in treating experimental patellar osteochondral defects might indicate a possible future application[149].

Mature cell dedifferentiation has been reported as a source of a pluripotent-like cell population. Mature adipocytes from WAT dedifferentiated *in vitro* by ceiling culture were found to revert to an undifferentiated phenotype and gain proliferative and differentiation capabilities[150]. Dedifferentiated fat (DFAT) cells have triploblastic differentiation potential *in vitro* and do not generate teratomas when injected into SCID mice[151]. DFAT cells are more homogenous than ADSCs and display mesenchymal surface markers and SSEA-3. DFAT was found to differentiate multiple cell lineages, including adipogenic, osteogenic, chondrogenic, myogenic, angiogenic and neurogenic lineages, and was tested in preclinical models of spinal cord injury rehabilitation of cardiac tissue after infarction[152,153]. DFAT cells were found to possess osteogenic capabilities when cocultured with periodontal ligament stem cells and might be a suitable cell source for periodontal regeneration[154]. DFAT cells display better differentiation capabilities, including osteogenic capabilities, than ADSCs from the same source. WAT-derived pluripotent cell populations are more homogenous than ADSCs and possess multilineage differentiation potential. Their prospective use for bone regeneration strategies is appealing and warrants more investigation.

FAT GRAFTING AND BONE HEALING

Not only cells but also WAT as a whole have been used in plastic and cosmetic surgery for aesthetic

reasons but also for supporting wound healing and skin support[155]. WAT has only recently been tested for its possible effect in supporting bone healing. Fragmented autologous WAT was shown to significantly increase mineralized matrix deposition in calvarial defects in rabbits compared to blood clot-treated and nontreated controls[156]. Fragmented WAT is investigated, as well as an autologous biomaterial that could be genetically modified to induce bone healing. In an *in vitro* study, genetically modified fragmented WAT overexpressing BMP-2 was shown to undergo mineralization in osteoinductive conditions[157]. The same team reported that the homodimer BMP-2 induced increased mineralization at lower doses compared to heterodimer BMP-2/6 or BMP-2/7; however, these findings need to be confirmed by *in vivo* studies[158]. WAT has been considered a modality to deliver microvascular grafts to healing bone defects to prevent atrophic nonunions. A thermoresponsive hydrogel (TRH) was used as a delivery system for WAT microfragments. However, local delivery of fragmented WAT-loaded TRH impaired bone formation in a murine model of bone defects, even though vascularization was improved. This undesirable outcome was thought to be produced by reduced VEGF expression in early phase bone healing, stressing the need for stage-specific delivery of bioactive factors [159].

CONCLUSION

Despite consistent research in recent decades, few clinical trials have tested the use of AT- or AT-derived cells for bone regeneration. To date, no clinically approved engineered product or cell therapy exists for treating impaired fracture healing, osteoporosis or ONFH. Particular challenges regarding cell heterogeneity and the type of cell used for different bone regenerative strategies are adding to the general challenges encountered by the development and approval of any advanced therapeutic medicinal product (ATMP). ADSC stemness characteristics are donor-dependent. The age of the donor has been thought to influence the quantity and quality of mesenchymal progenitors derived from WAT; however, conflicting reports exist in this respect. A decreased yield of SVF and ADSC colony-forming units per tissue, increased mitochondrial ROS production and impaired migratory and differentiation potential were reported for elderly donors in some studies[160,161]. Other studies, however, report similar characteristics of ADSCs derived from donors ranging from 8-62 years of age confirmed in a clinical case series where ADSCs were used for treating bone nonunions in combination with osteoconductive grafts[162]. The differences might be explained by the fact that studies reporting impaired ADSC characteristics in elderly individuals do not elucidate their possible coexisting diseases (such as diabetes, metabolic syndrome, and obesity) ADSCs derived from T2D patients were found to possess reduced viability and proliferative potential, exhibiting mitochondrial dysfunction and a senescence phenotype due to excessive mitochondrial ROS accumulation[163]. The T2D ADSC secretome was also modified with reduced VEGF, adiponectin, and chemokine (C-X-C motif) ligand-12 secretion and overproduction of leptin[164]. ADSCs derived from obese donors displayed reduced proliferative and differentiation potential compared to ADSCs from normal BMI donors. Obese ADSCs were shown to induce a proinflammatory phenotype in murine Mcfs and microglia, increasing the expression of proinflammatory genes and nitric oxide pathway activity while impairing their phagocytosis and migration[165]. Metabolic syndrome and T2D ADSCs have increased susceptibility to apoptosis and senescence with increased expression of senescence-associated β-galactosidase, a high level of anti-apoptotic protein B cell lymphoma-2 and decreased expression of the marker of proliferation Ki-67. These changes result in decreased proliferation, morphological changes with enlarged cellular bodies and nuclei and increased apoptosis of ADSC factors that affect the stemness of ADSCs derived from these donors[166]. WAT status obesity and weight loss, age and disease-related lipotrophy affect the quantity and quality of SVF and ADSCs that can be derived from autologous sources.

These findings underscore the need for thorough characterization of cells before their use for certain prospected clinical applications. Genomic and proteomic profiling of the ADSC phenotype, as well as their secretome, could identify biomarkers for selecting the appropriate cell source for a particular application in bone healing. This would result in possible test panels for determining whether autologous or allogenic cell sources are the best choice for the desired outcome. Modelling the desired profile for a specific application in bone healing (such as osteogenic potential and trophic and/or anti-inflammatory effects) would help select the cell phenotype that is more suitable for bone tissue engineering or cell therapy for fracture healing or other bone-specific diseases. Cell profiling for a projected ATMP would positively impact product characterization, standardized manufacturing and quality control.

Expanding the use of pluripotent cells from WAT, MUSE and DFAT cells, which are less donor-dependent and have increased osteogenic potential, could increase the chance for successful bone engineering strategies. Given the capability of MUSE cells to traffic, home and differentiate at the site of injury, a combined acellular scaffold with systemic or local MUSE delivery could represent a convenient modality for bone grafting and fracture healing. An important gap of knowledge still exists regarding the mutual interrelation between different AT types and bone in its normal and pathological states. Not only AT but also bone metabolism, fracture and the modality of fracture treatment can influence AT

locally and systemically. BMP-2 treatment of long bone fractures in high- and low-fat diet-fed mice was shown to display increased vessel parameters and femoral adipocyte numbers irrespective of diet. Local BMP-2 delivery was shown to exert a diet-dependent effect on lung endothelial and bone marrow endothelial cells, influencing gene expression and *in vitro* tube formation capabilities[167]. These findings point out the necessity to investigate the complex interrelation between AT and bone from a systemic perspective. The role of BMAT in orchestrating local and systemic bone metabolism and bone healing and its interrelation with WAT and BAT need further investigation. Two recently registered clinical trials are salutary in this respect, as they are poised to compare WAT and BMAT characteristics in postmenopausal and osteoarthritic subjects (NCT03678831), as well as to model the complex interrelation between BMAT adipocytes and osteoblasts derived from osteoporotic patients (NCT04377880) (Table 2).

Multiple omics profiling of various cell populations and modelling their interactions in silico and *in vitro* will increase the understanding of intricate factors that govern AT and bone balance. The increased availability of organoids and organs on chip technologies that enable high-throughput experiments will enable the validation of computer models. These models will derive improved therapeutic targets for treating bone diseases and impaired fracture healing, as well as methods for using preventive measures for maintaining health in both compartments.

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REVIEW

Application and prospects of high-throughput screening for *in vitro* neurogenesis

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Abstract

Over the past few decades, high-throughput screening (HTS) has made great contributions to new drug discovery. HTS technology is equipped with higher throughput, minimized platforms, more automated and computerized operating systems, more efficient and sensitive detection devices, and rapid data processing systems. At the same time, *in vitro* neurogenesis is gradually becoming important in establishing models to investigate the mechanisms of neural disease or developmental processes. However, challenges remain in generating more mature and functional neurons with specific subtypes and in establishing robust and standardized three-dimensional (3D) *in vitro* models with neural cells cultured in 3D matrices or organoids representing specific brain regions. Here, we review the applications of HTS technologies on *in vitro* neurogenesis, especially aiming at identifying the essential genes, chemical small molecules and adaptive microenvironments that hold great prospects for generating functional neurons or more reproductive and homogeneous 3D organoids. We also discuss the developmental tendency of HTS technology, *e.g.*, so-called next-generation screening, which utilizes 3D organoid-based screening combined with microfluidic devices to narrow the gap between *in vitro* models and *in vivo* situations both physiologically and pathologically.

Key Words: High-throughput screening; Stem cells; Neurogenesis; Cell differentiation; Three-dimensional cell culture; Cellular microenvironments

Core Tip: High-throughput screening (HTS) is a promising technology that can screen out targets from thousands of candidates. Here, we review the evidence that HTS could be beneficial in neurogenesis methods in various ways: The HTS method can screen out specific genes that induce neural induction, small molecules that facilitate neural differentiation, and three-dimensional microenvironments that could better modulate the microenvironments *in vivo*. We also focus on the application and prospects of HTS in *in vitro* neurogenesis, as organoid-based and microfluidic platforms are needed for future research.

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INTRODUCTION

High-throughput screening (HTS), also called large-scale cluster screening, first appeared in the 1980s and utilized microplates as a platform, an automated handling system as an operator, and a variety of highly sensitive detection instruments to screen out "hits" from thousands of drug candidates. As an integrated and multidisciplinary technology, HTS combines diverse fields, such as molecular biology, medicinal chemistry, mathematics, computer science and microelectronic technology. With its rapid, efficient, economic, microscale, highly automatic and computerized features, HTS technology has made great contributions to biomedical research, such as identifying new drug candidates for pharmacological research[1,2], enzyme engineering, including the directed evolution of enzymes[3], and genetic research[4].

Based on ligand-target interactions, HTS can be performed between various candidates and targets, including substrates and enzymes, inhibitors and enzymes, ligands and receptors, proteins and proteins, and DNA and proteins[5]. According to the *in vitro* screening models, HTS can be mainly divided into cell-free assays, also called biochemical assays, and cell-based assays. While cell-free assays dominate in the early stage of HTS, cell-based assays are gradually gaining an essential role because some cellular processes, such as transmembrane transport, cytotoxicity effects or other off-target effects, can be tested in cellular models, and some screening targets are difficult to extract and purify from cells [6]. Significantly, in recent studies, screening targets have been extended from biochemical compounds such as enzymes, receptors, antibodies, nucleotides and living cells to tissues and even organoids to investigate protein–protein/DNA/RNA interactions, cell-protein interactions, cell–cell interactions and even protein-tissue interactions. Therefore, categories of testing candidates are also developing from biochemicals aiming at diverse targets such as enzymes and receptors in intracellular signaling pathways to microenvironments that are suitable for various cellular behaviors. Since the exploration of cell-extracellular matrix (ECM)-interactions is growing and three-dimensional (3D) cell culture technologies are developing, the HTS platform is evolving from two-dimensional (2D) to 3D. In previous research, hydrogel droplets and synthetic scaffolds could be attached to HTS platforms[7].

In addition to extending the variety of screening targets and candidates, researchers have also been working on improving the miniaturization, integration and automation of the screening platform to meet the increasing need for HTS applications in biomedical research. Specifically, the screening platform has developed from comprising microtiter plates with 96 wells to those with 384 wells and then to those with 1536 wells[8]. To further elevate the screening efficiency, microarrays are utilized to promote integration by immobilizing protein or DNA targets on glass chips. Then, to separate each spot, save reagents and create various cellular microenvironments, combinations of microwells and micropillars are applied for HTS[9,10].

Furthermore, microfluidic-based microarrays also play an important role in HTS because of their higher efficiency, improved automation, controlled microenvironments, adjustable flow parameters, achievement of microscale reaction volumes and the capacity for single-cell analysis. Methods based on microfluidic systems can be divided into two groups: Droplet-based microfluidics and array-based microfluidics[11]. Assay-based microfluidic devices have also been successfully utilized in HTS for drug screening[12,13], cell heterogeneity analysis[14], cell–cell interactions[15] and even cell–ECM interactions[15].

Compared with array-based microfluidic devices, droplet-based microfluidic devices are well suited for analyzing single-cell activities because biomolecules, particles or even single cells can be encapsulated in water-in-oil droplets that are emulsified when they are flowing through the droplet-producing devices to form the droplet library[16]. Every droplet contains a barcode that represents the elements encapsulated. The barcodes usually include nucleic acid sequences, which are capable of large screens, and fluorescent tags, which are suitable for real-time reading[17]. The droplets pooled in the library are then reinjected into the microfluidic device, usually merging with other cells or biomolecules

to start the incubation, followed by a screening based on various characteristics, such as cell density[16] and fluorescence intensity[18]. In addition, droplets can also be sorted according to the variety of readouts. The strategies applied in droplet sorting are based on fluorescence-activated cell sorting (FACS), which is a mechanical actuation, also called reverse cell sorting, accomplished with the assistance of peristaltic pumps and valves[19] as well as dielectrophoresis[20].

Over the years, the requirements of neurogenesis methods have grown with the increase in neurodegenerative diseases, and *in vitro* neurogenesis has been playing an important role in disease modeling, tissue engineering, drug screening and regenerative medicine[21-25]. However, the ways to generate mature and functional neural cells with high efficiency and cell purity remain a problem. Here, we discuss how HTS technology promotes the progression of *in vitro* neurogenesis in three sections, including screening out functional genes regulating neurogenesis, small molecules inducing neural lineage cells, and suitable microenvironments that facilitate *in vitro* neurogenesis. Furthermore, we review the applications of these obtained neural lineage cells using HTS technologies. Finally, with this review, we strengthen the connections between this promising and fast-developing technology and *in vitro* neurogenesis to raise awareness of generating more functional, mature and specific neural cells, as well as reproducible and standardized organoids with HTS technologies, for the sake of establishing robust neural developmental or disease models to better serve drug screening and regenerative medicine.

FABRICATION OF THE HTS PLATFORM

For the sake of designing various patterns for microarrays and microfluidic chips, depositing targets on the substrate surface is an essential step, which has been performed using a variety of methods, such as direct contact printing and noncontact printing, also known as ink-jet printing, photolithography, soft lithography, electron beam lithography, nanoimprint lithography, dip pen nanolithography, and laser-guided direct writing[26]. Direct contact printing can place desired biomolecules as ink from a stamp or a pin, linked to a high-precision robotic arm, to substrates with a reactive surface, which is usually accomplished by click reactions[27]. Noncontact printing can eject samples in the form of droplets to specific positions mainly by piezoelectric and thermal printers[26]. Photolithographic techniques can immobilize biomolecules on a substrate with photosensitive groups, for example, self-assembled monolayers such as alkanethiol[28], as linkers[29]. Patterns on the microarrays can be designed according to the patterns on the masks, which could selectively activate the photosensitive groups with UV light irradiation[29], and then the solubility of the photoresists will change, leaving the substrate in the development step. For soft lithographic techniques, the word "soft" can describe elastomeric stamps or channels, which are made of commonly used poly(dimethylsiloxane) (PDMS)[30]. PDMS stamps are utilized in microcontact printing, while channels are required in microfluidic channel flow patterning; these are the main methods used in soft lithographic techniques. The PDMS stamp can be fabricated using photolithographic techniques as the master is patterned with UV light and photoresists on the substrate, and then the liquid prepolymer is cast on the prepared master to form elastomeric stamps [30]. After that, these stamps can pattern the substrates through microcontact printing using molecules that can interact with biomolecules and cells.

To further enhance the resolution to the nanoscale for higher throughput, electron beam photolithography and dip pen nanolithography are applied for direct protein patterning on microarrays[31-35]. Nanoimprint lithography is also a nanostructure patterning technique that has been used to manipulate multiarchitectural chips with fields of topographies in nanometer dimensions to carry out high-throughput analysis for the screening of topographical structures that could promote stem cell differentiation[36,37]. In addition to using biomolecules as targets, cells can also be directly patterned into substrates, although attaching them to biomolecules that have been positioned to substrates is another matter. This technique is called laser-guided direct writing, in which the laser, focused by the lens, propels single cells with optical forces toward the substrate to form cell clusters[38]. This technique has been applied in tissue engineering through the reconstruction of tissues by micropatterning cells on soft matrices such as collagen or Matrigel to build cell–cell interactions that resemble those in the native microenvironment[39].

CURRENT *IN VITRO* NEUROGENESIS METHODS

For neurobiological research, *in vitro* neurogenesis plays a significant role in conducting drug screening, establishing models for investigating mechanisms of neural development or diseases, and deepening research on regenerative medicine for cell therapy and tissue engineering[22,23,40]. Consequently, exploring more efficient methods for *in vitro* neurogenesis, including obtaining pure and functional neurons, building neural circuits, and forming neural tissue and even cerebral organoids, is of vital importance. To date, various methods have been used to manipulate *in vitro* neurogenesis (Figure 1). Embryonic stem cells (ESCs), pluripotent stem cells (PSCs) and mesenchymal stem cells (MSCs) have

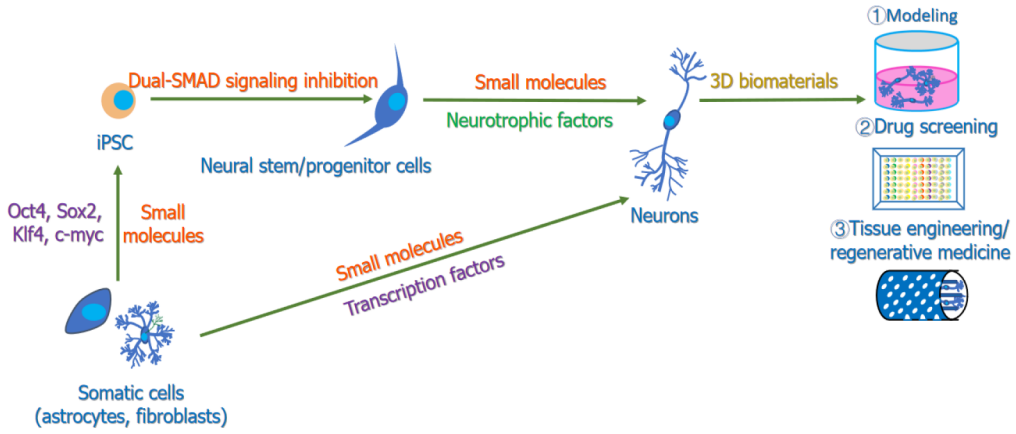

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Figure 1 Main protocols and applications of *in vitro* neurogenesis. Neurons can be generated *in vitro* through the differentiation of induced pluripotent stem cells or the transdifferentiation of somatic cells with the aid of transcription factors, growth factors or small molecules[51,131]. With the combination of 3D biomaterials, induced neurons can be applied in various fields, including modeling, drug screening, neural tissue engineering and regenerative medicine[213,232,276]. iPSCs: Induced pluripotent stem cells.

been induced to differentiate into functional neuronal cells through growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-1) as well as neurotrophic factors such as neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) (Supplementary Table 1)[41-43]. Sometimes, to obtain specific subtype neurons, key factors participating in subtype neuronal development are added; for example, sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8) are reported to be essential in the induction of dopaminergic neurons[44,45].

Furthermore, it has been found that the forced expression of distinct neurogenesis fate determinants, as well as the help of growth factors, can support the differentiation of ESCs and PSCs toward neural lineage cells (Supplementary Table 1)[46-49]. In fact, since induced PSCs (iPSCs) were developed by the integration of four transcription factors[50], neural transcription factors have been an efficient tool to reprogram fibroblasts[51,52], astrocytes[53,54] and other cell lines[55] to neurons *in vitro*. Recently, with the assistance of HTS, researchers have successfully explored some small molecules and their combinations to promote neural differentiation[56,57] and cell reprogramming[58-61] to obtain functional neurons (Supplementary Table 1). In these ways, functional and pure neurons with different neural subtypes, including dopaminergic neurons, cholinergic neurons, GABAergic neurons and glutaminergic neurons, have been generated.

Over the years, 3D culture systems have been developing rapidly, as they can provide cells with mechanical support, external stimuli and cell–ECM interactions to better mimic the architecture and functions of the *in vivo* microenvironment in living tissues and organs compared with conventional 2D cultures[62]. For many years, adding 3D matrices has become an attractive choice for the culture of neural cells and the generation of neural tissues or even neurospheres *in vitro*[63-65]. For cell culture, the three most commonly used models include the "on-gel model", the "sandwich gel model" and the "in-gel model". For even more complex 3D culture models, a study has cocultured predifferentiated human PSC (hPSC)-derived astrocytes with hPSC-derived, Ngn2-induced neurons on Matrigel-coated glass chips and observed more mature morphology of astrocytes, followed by the coculture of astrospheres and neurospheres on polytetrafluoroethylene membranes as organotypic-like cultures, and the astrocytes improved morphological complexity with fine leaflet-like structures[66].

There have also been other 3D culture systems containing spinner flasks and rotary bioreactors that can prevent cell attachment to the surface and aid in generating 3D spheroids[67], such as neural organoids. Over the past decade, organoid technologies have become a promising method to bridge the gap between cellular models and animal models and to better recapitulate the complexity of the cytoarchitecture at the tissue/organ level. The technology relies on the self-organization, self-renewal and differentiation capacity of ESCs or iPSCs and their potential to form cells from three germinal layers [68]. The protocol for generating cerebral organoids from hPSCs mainly includes the generation of embryonic bodies, the neural induction period, the neural differentiation period embedded in Matrigel, and the procedure for brain tissue growth and expansion in spinning bioreactors to provide enough oxygen[69,70]. Therefore, with the supplementation of 3D matrices and specific biomolecules, including growth factors and small molecules that precisely control the signaling pathways regulating neural lineage induction and neural differentiation, as well as devices providing sufficient oxygen for tissue growth, cerebral organoids containing discrete brain regions[69] and even organoids with specific brain regions, such as forebrain organoids[71,72], midbrain organoids[73,74], thalamic organoids[75] and cortical organoids[76], can also be generated. Specifically, to imitate morphogen concentration gradients

in vivo, an inducible Shh-expressing hPSC line was constructed and embedded in one pore of developing organoids to create a Shh signaling gradient[72]. Treatment with LDN193189, SB431542 and XAV939 inhibited bone morphogenetic protein (BMP), transforming growth factor (TGF)-β, and Wnt signaling, respectively, to induce organoids toward forebrain identity[72,77]. Midbrain organoids were also generated based on the mechanism of regional patterning, as the addition of FGF8 and activation of Shh signaling have been proven to be significant elements in inducing midbrain dopaminergic identity [73,74,77,78]. Cortical organoids are induced through dual-SMAD signaling suppression following the induction of cortical identity patterns *in vivo*[76,77].

However, the self-organization ability of PSCs usually leads to an unpredictable arrangement of the internal structure of the organoids, which increases the uncertainty of their applications in *in vitro* modeling. Therefore, to improve the reproducibility between batches, a feasible method is to control the initial culture conditions, such as the starting cell types, cellular density, 3D matrices, and size and geometry of aggregates, because minimal deviations from the initial conditions will generally result in batch-to-batch controlled organoids[79]. To achieve this, trials have been performed to engineer 3D matrices and explore more suitable scaffolds, such as poly (lactic-co-glycolic acid) (PLGA) microfilaments, for guiding self-organization[71].

APPLICATIONS OF HTSS TO NEUROGENESIS

Identification of functional genes regulating neurogenesis

During neurogenesis, genes correlated with neural development are expressed in spatial and temporal order[80]. In previous studies, key genes have been identified[81] and have recently been the focus of further trials seeking to generate functional neural cells[48]. Significantly, basic helix loop helix transcription factors play important roles in neural specification and differentiation[82], and the forced expression of transcription factors has been manipulated to convert ESCs, iPSCs or nonpluripotent somatic cells into functional neurons even with specific subtypes, including midbrain dopaminergic neurons, spinal motor neurons, and forebrain GABAergic interneurons[21,51,83-86]. In addition to transcription factors, miRNAs are also effective tools to induce neural cell types; for example, Yoo *et al* [87] reported that miR-9/9* and miR-124, together with NeuroD2, could convert human fibroblasts into functional neurons by regulating SWI/SNF-like BAF chromatin-remodeling complexes.

For decades, functional gene identification has been carried out based on forward genetic approaches, mostly through whole-genome mutagenesis screening. Chemical mutagens, such as ethylmethanesulfonate and ethylnitrosourea, as well as polymerase chain reaction-based gene deletion strategies, are commonly applied to induce mutations, and phenotypes are evaluated to screen out functional genes [88-91]. However, for neurogenetic research, this method usually requires model organisms, including transgenic mice, Drosophila or zebrafish, which makes it time-consuming and difficult to manipulate screening on high-throughput platforms. Reverse genetic approaches are also becoming popular strategies to perform HTS to identify functional genes controlling various cellular behaviors. Since the discovery of RNA interference (RNAi), RNAi-based HTS has shown superiority in the identification of functional genes, the dissection of signal transduction pathways, and target exploration for drug development[92,93]. Compared with traditional forward genetic methods, RNAi screening fits more to cell-based screening, which is more suitable for conducting experiments at the cellular and molecular levels and is easier to utilize on HTS devices. Due to its advantages, this technique has been widely applied to identify genes that regulate neural development. Koizumi *et al*[94] injected double-stranded RNA into the preblastoderm embryos of Drosophila and found 22 genes that influence embryonic nervous system development, seven of which had unknown functions, nine of which had known functions, and the rest of which had known nervous system development phenotypes, such as dmt expressing a nuclear localization motif for peripheral nervous system development. Güneş *et al*[95] performed a selection-based screening and transduced $CD34^+$ hematopoietic stem and progenitor cells and neural stem cells (NSCs) with shRNA. After next-generation sequencing and the comparison of shRNA representation in two cell types, they determined SMARCA4 to be the stemness regulator that controls NSC self-renewal by upregulating RE-1, a silencing transcription factor, and downregulating BAF53, suggesting its function in the repression of cell differentiation toward the neural lineage[95]. Other RNAi screening studies have also identified genes that play roles in neural outgrowth, axonal regeneration and neural cell death[88,96,97].

Over the years, with the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) system in prokaryotic organisms, CRISPR/Cas9 has opened new avenues toward HTS. The method of CRISPR-based HTS can be divided into arrayed/plate screens and pooled/barcode screens [4]. In arrayed screen format, the process is conducted in multiwell plates, and sgRNAs are individually delivered through viral transduction or transfection, followed by screen readout through high-throughput imaging, while for pooled screens, sgRNAs are synthesized and delivered as a pool, followed by viability screening through next-generation sequencing to evaluate the difference in the abundance of sgRNAs between samples for the identification of target genes or phenotypic screening, such as through FACS[4]. Compared with RNAi screening, CRISPR/Cas9-based screening has fewer

off-target effects and is capable of investigating nontranscribed spacers and noncoding RNA, as RNAi can suppress gene expression only at the posttranscriptional level, whereas CRISPR/Cas9 can invalidate target genes at the genetic level[98]. Thus, the applications of CRISPR/Cas9 screening have grown in recent years, and genes related to bacterial toxicity[99], DNA mismatch repair pathways[100], new drug targets[101], cell viability and proliferation[102] have been identified in human cancer cells.

Significantly, CRISPR activation (CRISPR/a) and CRISPR interference (CRISPR/i) also play important roles in HTS in the identification of functional genes. These methods have been reported to serve as an effective tool in developmental biology studies. For instance, Genga *et al*[103] combined CRISPR/i screening and single-cell RNA-seq and identified FOXA2, the transcription factor that plays a significant role in endoderm development, as inhibition of FOXA2 impaired differentiation toward the foregut endoderm and the subsequent hepatic endoderm. In a neural differentiation study, Liu *et al*[104] utilized CRISPR/a screening through the establishment of a sgRNA library consisting of 55561 sgRNAs targeting all computationally predicted transcription factors and other DNA-binding factors and identified various transcription factors that could promote neural differentiation by sorting Tubb3-hCD8$^+$ cells. They also studied the interactions between these hits through a combination of two sgRNAs and found that Ngn1, along with Brn2, Ezh2 or Foxo1, significantly improved the conversion efficiency of mouse embryonic fibroblasts (MEFs) into neurons[104]. The investigation of one of the hits, Ezh2, showed the downregulation of endodermal- and mesodermal-related genes in Ezh2-induced neurons, which indicates the possible mechanisms of enhanced neural differentiation of Ezh2[104].

Given that HTS possesses the ability to identify functional genes, particularly transcription factors related to neural development, future directions could still focus on the genetic networks of neurogenesis, especially the function of noncoding sequences, such as noncoding RNAs. For example, Zhu *et al*[105] designed a paired guide RNA CRISPR/Cas9 Library to delete approximately 700 long noncoding RNAs (lncRNAs) in the human liver cancer cell line Huh7.5OC. From a genome-scale lncRNA deletion screen, they found 9 lncRNAs positively or negatively correlated with the proliferation and survival of cancer cell lines[105]. Furthermore, CRISPR/a and CRISPR/i have also been proven to be useful in lncRNA screening. Liu *et al*[106] performed a genome-wide CRISPR/i screen with dCas9-KRAB targeting 16401 lncRNA loci in 6 transformed cell lines and an iPSC cell line, and the results showed that 499 lncRNAs participated in cell growth, such as LINC00263, the knockdown of which downregulated the proliferation of the U87 cell line and upregulated endoplasmic reticulum stress- and apoptosis-related genes. A CRISPR/a screen was also applied to identify lncRNAs related to the drug-resistance pathway in cancer cell lines[107]. In addition, CRISPR screening has been reported to discover functional miRNAs. Panganiban *et al*[108] conducted a genome-wide CRISPR/Cas9 screen and recently demonstrated that knockout of miR-124-3 led to upregulation of C/EBP homologous protein 10, the transcription factor associated with ER stress-mediated apoptosis, by regulating the IRE1 branch of the ER stress pathway. Moreover, screening out noncoding genes that serve as endogenous regulatory elements could help to further deepen our understanding of gene expression regulation. Klann *et al*[109] performed CRISPR/Cas9-based epigenomic regulatory element screening through dCas9-KRAB and dCas9-p300 to repress or activate the activity of the DNase I hypersensitive site and identified previously uncharacterized regulatory elements controlling the expression of b-globin and HER2. Baumann *et al*[110] strongly induced master transcription factor Sox1, as well as a neuroepithelial marker, through dCas9-VP64 to target the promoter of Sox1 and restored the neuronal differentiation potential of NPCs. Then, they transfected dCas9-Tet1 into NPCs that stably expressed dCas9-VP64 after transducing gRNA and found that the neuronal differentiation potency was increased with dCas9-Tet1 decreased DNA methylation levels around the transcription start of Sox1[110]. Thus, it is helpful to further understand the intrinsic interaction between activation of transcription factors and the regulation of epigenetic editing and even chromatin modification in neural development. Consequently, HTS could be a leading method to identify key genes and pathways, including transcription factors and noncoding regions related to neural development, which would provide novel targets for *in vitro* neurogenesis.

Identification of small molecules for neural lineage induction

Although the overexpression of transcription factors in initial cells has made great progress in the induction of functional terminal differentiated cells, it could lead to safety problems such as tumorigenesis when the viral vectors integrate into the genomes of receptor cells[111]. Compared with the forced expression of transcription factors, utilizing cell-permeable chemical small molecules is safer, more cost-effective, less time-consuming, and easier to standardize. Thus, developing small molecules that could replace the effect of transcription factor overexpression has great prospects. This assumption first came into reality when Hou *et al*[112] identified a combination of seven small molecules that could reprogram MEFs to ESC-like PSCs. Before that, when many studies focused on the identification of small molecules that are capable of replacing defined transcription factors, HTS technology made huge contributions to identifying those specific small molecules.

For HTS technology, cell-free assays have been of great support for the identification of small molecules based on their effects on activating or blocking signaling pathways that facilitate or inhibit neural differentiation, which usually act as agonists or antagonists of kinases belonging to those signaling pathways. For example, SB431542 is an inhibitor of the ALK5/TGF-β1 receptor, which was

identified by a flashplate-based assay with the immobilization of GST-tagged Smad3, and GST-tagged ALK5 was used as a kinase[113]. Except for using the interaction between biomolecules, especially between candidates and kinases or receptors affiliated with specific signaling pathways, cell-based methods are more commonly applied in screening compounds that could replace essential transcription factors, including in the period when the chemical induction method was the only method available for exploration. Takahashi and Yamanaka[50] screened out iPSCs by integrating a bgeo cassette, a fusion of the β-galactosidase and neomycin resistance genes, into Fbx15, a downstream gene of Oct4, to conduct iPSC screening through drug resistance to G418. However, iPSCs isolated in this manner were different from ESCs in their gene expression patterns, and screening was performed based on the activation of endogenous Nanog or Oct4 with a drug-resistance marker or the green fluorescent protein (GFP) reporter gene[114-116]. Recently, optical screening methods using fluorescent proteins, luciferase or the lacZ gene were proven to be effective in cell-based screening. To screen small molecules that could replace Sox2 in reprogramming, an Oct4-GFP transgenic reporter was used to provide Oct4-GFP+ colony numbers so that the reprogramming efficiency could be represented after retroviral transduction of MEFs with Oct4, Klf4 and c-Myc[117]. Then, from screening 200 small molecules, it was found that Repsox could substitute Sox2 even without c-Myc or the histone deacetylase inhibitor valproic acid (VPA), which could greatly improve reprogramming efficiency[118] through the inhibition of the TGF-β signaling pathway[117]. In addition, the G9a histone methyltransferase inhibitor BIX-01294 was also demonstrated to substitute Sox2 in the presence of Oct4 and Klf4 (c-Myc is dispensable when generating iPSCs from mouse and human fibroblasts)[119,120]. Other transcription factors, including Klf4, could also be replaced during reprogramming by VPA[121] or the GSK-3β or CDK inhibitor kenpaullone [122], and Oct4 could be replaced by the inhibitor of the ALK5/TGF-β1 receptor, SB431542 or Repsox [123].

In addition, HTS has been generally used to screen small molecules that could generate functional neurons to eliminate the ectopic expression of transcription factors. Li *et al*[59] screened approximately 5000 small molecules using Ascl1-infected mouse fibroblasts and found that forskolin, SB4315342, ISX9 and CHIR99021 could improve the number of TauEGFP-/Tuj1-positive neural cells. Subsequently, I-BET151 was screened out from approximately 1500 candidates in the presence of the former four small molecules and in the absence of the transcription factor Ascl1[59]. In subsequent tests, it was found that ISX9 was capable of activating the master neural genes NeuroD1 and Ngn2[59]. In addition, different combinations of various small molecules can also be screened to obtain more efficient cocktails for neural differentiation. Chambers *et al*[124] screened approximately 400 different combinations according to the day the compounds were added, and they confirmed that CHIR99021, DAPT and SU5402, which were added at day two of differentiation, along with SB431542 and LDN193189, could direct the differentiation of human NPC (hNPC) into nociceptive sensory neurons. They evaluated the decrease in PAX6+ cells and the improvement of TUJ1+ cells as the screening phenotype[124]. In another study, Zhang *et al*[61] performed a screen containing hundreds of combinations among 20 small compounds with different concentrations in a stepwise protocol and identified a cocktail including LDN193189, SB431542, TTNPB, thiazovivin, CHIR99021, VPA, DAPT, SAG, and purmorphamine that could convert human astrocytes into functional neurons.

To date, small molecules have been widely utilized in inducing neural cell lineages from PSCs or somatic cells, as a number of experiments have successfully generated functional neurons from ESCs, fibroblasts or astrocytes by chemical cocktails (Supplementary Table 2)[56,58-61,125,126]. Among the chemical compounds, SB431542 and LDN193189 have been widely applied to induce neuroectodermal cell lines from PSCs since the inhibition of dual-SMAD signaling was proven to be capable of converting human ESCs (hESCs) to PAX6+ neuroepithelial cells by the SB431542/Noggin protocol[127]. LDN193189 is an inhibitor of ALK2/ALK3 that was used to replace noggin, a soluble BMP antagonist. Dorsomorphin, also known as Compound C, was able to inhibit the BMP type 1 receptors ALK-2/ALK3/ALK6 and subsequently repress the phosphorylation of Smad1/5/8[128]. However, dorsomorphin resulted in moderate inhibition and unstable metabolism; thus, LDN193189 was recently developed and exhibited a highly decreased IC_{50}[129]. In addition, CHIR99021, also named CT99021, is an inhibitor of GSK3β and can activate Wnt signaling, which has been demonstrated to play an important role in maintaining neural stem/progenitor cell proliferation and differentiation[130]. Nevertheless, CHIR99021 has been reported to induce the neural crest lineage through activation of the Wnt signaling pathway[124], the inhibition of which by XAV939 could help to generate cortical neurons [131]. Of note, CHIR99021 was added in the final differentiation step, as it can function in the promotion of axonal outgrowth and synapse formation[131]. Significantly, Kirkeby *et al*[57] tested CHIR99021 in a dose-dependent manner and found that hESCs differentiated into neural progenitors with all regions from the telencephalon to the posterior hindbrain along the rostrocaudal axis following increasing concentrations of CHIR99021. Except for the rostrocaudal axis, dorsoventral axis patterning could also be induced by small molecules, as ventralization can be regulated by Shh, and dorsalization can be controlled by the Wnt canonical pathway and the BMP pathway[77]. Purmorphamine is a Smoothened receptor agonist that can activate the Shh signaling pathway and has a similar effect as another small molecule called SAG, which is a potent Smoothened receptor agonist. Thus, neural subtypes could be enriched through the coordination of Shh signaling, Wnt canonical signaling and BMP signaling[132]. Forskolin is also a commonly used small molecule that functions as a diterpene adenylate cyclase

activator, and the addition of forskolin could increase the level of intracellular cyclic AMP (cAMP). Significantly, the activation of cAMP/PKA-cAMP-responsive element binding (CREB) signaling, for example, by treatment with dibutyryl-cAMP, can phosphorylate CREB protein, which is an essential transcription factor regulating many target genes related to the survival, proliferation and differentiation of neurogenic cells, such as Bcl-2, BDNF, tyrosine hydroxylase and somatostatin[133]. Furthermore, SU5402 and PD0325901 are inhibitors of FGFR1 and mitogen-activated extracellular activated signal-regulated kinase (MEK), respectively, which all serve as inhibitors of FGF/MEK/ERK signaling. DAPT, a g-secretase inhibitor, is likewise a small molecule commonly applied as a Notch signaling inhibitor. There have been a number of studies using the inhibition of FGF and Notch signaling to suppress cell proliferation and thus lead to differentiation[124,126,131].

To date, with the support of the HTS method, many types of small molecules have been developed to convert pluripotent cells such as ESCs and NPCs or nonneural somatic cells such as fibroblasts into neural lineage cells. However, there is still great demand to generate mature neurons with specific neural subtypes and positional cues of different brain regions. To meet this requirement, more selective chemical compounds with optimum concentrations and combinations with different addition orders are desired. Maury *et al*[134] utilized the automated 384-well plate format to treat hNPCs with various concentrations, durations, and combinations of small molecules and directed NPCs to spinal motor neurons and cranial motor neurons with specific regional identities. Additionally, developing diverse types of small molecules that regulate gene transcription through different mechanisms could be another perspective to expand the collection of small molecules for neural lineage conversion. In addition to small molecules that work as signaling pathway modulators, other chemical compounds that can repress epigenetic-related enzymes, such as histone-modifying enzymes, DNA methylation-associated enzymes, and modulate nuclear receptors remain to be further explored[135]. For example, VPA, an inhibitor of histone deacetylase; RG108, an inhibitor of DNA methyltransferases; and TTNPB, a retinoic acid (RA) analog and a nuclear receptor RAR agonist, are applied in the conversion of neural cells through transdifferentiation methods[58,61,126,136]. Therefore, further investigations of epigenetic mechanisms and the orchestrated signaling processes underlying neural lineage specification are required to develop targeted small molecules.

Screening for more suitable microenvironments for in vitro neurogenesis

The cell microenvironment consists of ECM, soluble molecules such as cytokines and hormones, and interactions with adjacent cells. To better facilitate neurogenesis *in vitro*, screening and reconstituting suitable microenvironments similar to those *in vivo* are important. However, traditional culture systems cannot be used to assess various microenvironmental factors at the same time. With the HTS platform, it is much more convenient and efficient to evaluate various parameters, including the type, topography, and stiffness of 3D materials and the types and concentration gradients of soluble biomolecules on a highly integrated chip.

Screening for 3D scaffolds: ECM molecules, mainly containing laminin, fibronectin, collagen IV, entactin, elastin, heparan sulfate proteoglycans, hyaluronan, chondroitin sulfate proteoglycans and tenascin-R in the CNS[137,138], are essential components of the microenvironment. In the natural matrices above, the widely applied 3D matrices are collagens[63,64,139,140], hyaluronan[141], and another commonly applied biomaterial called Matrigel[22,142,143] due to their superior contributions to the *in vitro* neural proliferation, differentiation and outgrowth of NSCs, NPCs or ESCs, some of which also build neural circuits and recapitulate CNS neural development.

Since these natural matrices are largely extracted from animals or cultured cells, it is difficult to control the biochemical and mechanical cues of batches[62]. Thus, to increase the reproducibility and reliability in further applications, synthetic scaffolds such as synthetic polymer hydrogels, which mainly contain self-assembling peptide hydrogels[144,145], poly(ethylene glycol) (PEG)[146-148], poly(lactic acid)[149,150], PLGA[151,152] and electrically conductive polymers including poly(pyrrole)[153-155], and carbon nanotubes[156,157] have become attractive tools for 3D *in vitro* neurogenesis (Supplementary Table 3). Although there are various kinds and different combinations of biomaterials to utilize [158], it would be helpful if we make the best use of HTS techniques to make comparisons of which kinds or combinations of biomaterials will be the priority for cell culture. Therefore, in future studies, it will still be quite important to explore recipes of biomaterials that work as effectively as possible for neurogenesis *in vitro* through 3D cell culture.

Microarrays based on glass slides on 2D platforms have been typically used for biomaterial screening, usually including polymer microarrays for screening synthetic polymer scaffolds and ECM or tissue microarrays for screening naturally sourced matrices. As mentioned before, biomaterials are patterned on slides through contact printing, injection printing and photolithography[159]. For polymer microarray screening, Anderson *et al*[160] fabricated a nanoliter-scale polymer array on which there were 576 different acrylate-based polymers in triplicate, synthesized by diverse combinations of 25 kinds of monomers through a light-activated radical initiator and UV light, attached to a poly (hydroxyethyl methacrylate)-coated slide. This microarray was used to evaluate polymer-based biomaterials for hESC attachment, spreading, proliferation and differentiation into cytokeratin-positive cells[160]. Using a similar method, this team subsequently constructed another polymer microarray with 1152

different combinations of 24 polymers mainly containing different forms of PLGA in triplicate[161]. They seeded human MSCs (hMSCs) and found that biomaterials containing PLGA-PEG inhibited cell attachment and spreading, while PLGA containing L-lactide could relieve this inhibition[161]. Recently, they carried out a screening with 22 acrylate monomers copolymerized in different combinations to generate 1488 arrays and tested their effects on hESC self-renewal[162].

In addition to polymer-based arrays, ECM microarrays are also commonly utilized for the investigation and dissection of functional elements for regulating cell behavior. Nakajima *et al*[163] displayed ECM-based biomaterials, including collagen I, collagen IV, fibronectin and laminin, as well as artificial biomaterials containing acidic gelatins, basic gelatins, ProNectin™ F plus and ProNectinTM L, poly(L-lysine), and poly(ethyleneimine) with weight-averaged molecular weights of 800, 10000, 25000, and 750000 on gold-coated glass plates. The results showed that fibronectin, laminin, Pro-F, Pro-L and PEI-0.8 could support NSC adhesion, while collagen and gelatins had no effect on NSC adhesion[163]. The probable reason for these results could be that NSCs adhere fibronectin and laminin through b1 integrin, and an electrostatic interaction might have promoted NSC's adhesion to PEI-0.8[163]. Ahmed *et al*[164] screened 190 combinations of 19 ECM proteins that were selected according to their expression in the ventral midbrain during dopaminergic neurogenesis and identified that Sparc, Sparc-like (Sparc-l1) and Nell2 could synergistically increase the number of TH^+ neurons differentiated from long-term neuroepithelial stem cells.

In addition, tissue matrix-based microarrays were fabricated by removing soluble components and mechanically fragmented matrices from 11 different porcine tissues and organs, which could preserve the natural diversity and complexity of biomaterials[165]. In this way, studies could focus on naturally sourced ECM components from various tissues and organs and analyze the tissue/organ-specific differences that subsequently lead to cell lineage specification. While retaining the complexity of ECM proteins, disassembling functional domains could also be an effective approach. Lin *et al*[166] conducted a peptide microarray and seeded normal murine mammary gland cells and demonstrated that the peptides LTGKNFPMFHRN and MHRMPSFLPTTL could induce epithelial-to-mesenchymal transition and decrease E-cadherin levels.

Screening for surface topography and morphology: In addition to the type of biomaterial, surface topography and morphology can also support neural induction. Studies have been performed to investigate the impact that continuous, discontinuous and random topographies that biomaterials have on the guidance and outgrowth of axons and dendrites[167]. In particular, continuous topographies can impact the orientation and shape of NSCs through the regulation of cytoskeleton rearrangement and nucleus elongation, while discontinuous isotropic topographies are reported to induce NSCs to the glial lineage[168]. Thus, to explore more suitable biomaterials expected to support neural growth, precise surface topologies were screened to search for the topologies that promote axon and dendritic growth. Large-scale screening showed that anisotropic grating patterns could best facilitate axon growth, while dendrites showed almost no sensitivity to surface topologies[169]. Furthermore, matrix stiffness also plays an unignorable role in neural development, as distinct subtypes of neurons exhibit different neurite outgrowth rates when cultured in conditions of varying elasticity[170]. To investigate the mechanical properties suitable for controlling specific cellular behavior, HTS has been utilized; for instance, Kumachev *et al*[171] constructed a droplet-based screening platform by encapsulating mESCs into agarose microgels with different elastic moduli. Kourouklis *et al*[172] arrayed various combinations of five ECM proteins on a poly(acrylamide) hydrogel substrate with three different elastic moduli to assess the effect of substrate stiffness on the differentiation of bipotential mouse embryonic liver progenitor cells.

Screening for combinations of growth factors: Soluble bioactive molecules such as growth factors, including bFGF and EGF; members of the neurotrophin family, including BDNF and GDNF; and members of the TGF family are important parts of the microenvironment during neurogenesis, regulating neural proliferation and differentiation[173-175]. The HTS method could be used to evaluate the best candidate or the best combination to promote *in vitro* neurogenesis. Konagaya *et al*[176] immobilized five growth factors, including bFGF, EGF, IGF-1, BDNF, and ciliary neurotrophic factor (CNTF), on a chip and displayed them either as a single component or as the combination of any of two factors to explore their function on NSCs. They found that either bFGF or EGF alone could facilitate the proliferation of NSCs, and that the combination of these two factors showed a synergistic effect. Both IGF-1 and BDNF could facilitate NSC differentiation toward the neural lineage, but CNTF promoted glial lineage differentiation[176]. Nakajima *et al*[163] used a cell-based assay to coimmobilize growth factors and natural or synthetic matrices, and they found that EGF promoted the maintenance of NSCs and that two nerve growth factor (NGF) family members, NGF and NT-3, could facilitate NSCs toward neuronal differentiation. In addition to for cell-based microarrays requiring immobilization of biomolecules, Muckom *et al*[177] applied a high-throughput microculture system consisting of complementary micropillars and microwells that could hold 532 independent microenvironments for cell culture. They seeded adult rodent NSCs and provided 6 soluble factors, BMP4, TGF-β, FGF-2, shh, Wnt-3a and Ephin-B2, and evaluated the extent to which their individual signals and double, tertiary and quaternary signal combinations could influence neural differentiation[177]. Their results indicated that

Wnt-3a and Ephin-B2 synergistically facilitated neural differentiation and maturation, while TGF-β, FGF-2 and Wnt-3a affected NSC proliferation and differentiation antagonistically[177].

Screening for 3D microenvironments: Beyond exploring single variants such as the abovementioned ECM proteins, surface topography, matrix stiffness and soluble factors, it could be more effective to combine various elements together and screen the whole microenvironment (Supplementary Table 4). A typically used approach is to premix ECM proteins and soluble signaling molecules in different combinations within multiwell plates, such as 384-well plates, and then to codispense the mixtures on substrate slides of the microarray. Later, cells are seeded on each spot. Lin *et al*[178] designed a microarray capable of screening microenvironments, including substrate stiffness, ECM matrices, various growth factors and cytokines. The elastic modulus could be adjusted by altering the base/cure ratio of PDMS to mimic hard tissues such as cartilage, cornea, and arterial walls, while regulating the acrylamide/bis-acrylamide ratio of PA could mimic soft tissues, including the brain, liver, and prostate[178]. Soen *et al*[179] also constructed a microarray for screening out microenvironments from 44 combinations of ECM proteins and signaling factors that promote the neural differentiation and specification of primary human NSCs (hNSCs). Moreover, Brafman *et al*[180] designed a 3D microarray screening method called arrayed cellular microenvironments, which could hold 8000 spots to screen microenvironments containing ECM proteins, growth factors and small molecules for evaluating cell attachment, growth and proliferation of hPSCs. To avoid interference between each spot on microarrays and to make the microculture system more suitable for culturing nonadherent cells, Gobaa *et al*[181] constructed arrays of PEG hydrogel microwells that could hold 2016 microenvironments to evaluate the effects of modular stiffness, bioactive molecules and ECM proteins on stem cells. The microwells were fabricated by stamping a silicon substrate with previously spotted biomolecules on a hydrogel substrate with different PEG concentrations to alter stiffness[181]. From screening these artificial niches, they studied the impact of laminin-1 and Jagged-1, the Notch ligand, on NSC fate[181].

However, when the microenvironment-based arrays are carried on a 2D platform, the cell–ECM interactions are weakened. For the sake of recapitulating the microenvironments and simulating the cellular states *in vivo*, 3D-based HTS platforms are recommended to evaluate the microenvironments proper to activate the specific cellular activities. Various microscale 3D culture screening systems mainly contain hanging drop plates, cellular microarrays and microwell plates (Supplementary Table 4)[67]. Because hanging drop plates are not suitable for long-term culture, these platforms might be more suitable for neural differentiation or organoid formation. Cellular microarrays, as well as microwell plates, are more broadly used for 3D HTS protocols, as these two methods allow for longer culture periods and more stability than hanging drop plates. For cellular microarrays, cells could be premixed with gels and coprinted on the substrates with robotic arrayers (Figure 2). For instance, Fernandes *et al*[182] designed a dual-slide incubation method that included a methyltrimethoxysilane-coated glass slide with preprinted all-trans-RA and FGF-4 and another poly(styrene-co-maleic anhydride)- and a poly(L-lysine)-coated glass slide containing mESCs embedded in alginate spotted with a robotic spotter. Another common platform contains two complementary chips, a microwell and a microchip. The procedure involves spotting the mixture of cells and 3D matrices on the top of the micropillar and adding culture medium with screening candidates, such as small molecules and soluble factors, into the microwell. Then, by stamping and incubating two complementary slides, the small molecules and growth factors can diffuse into the cell spots and trigger biological reactions (Figure 2). In this way, multiple elements in microenvironments can be screened to study the regulation of stem cell fate[183-185]. Given that microarray-based screening can result in interference between spots, microwell plates are also commonly used in 3D screening methods. Ranga *et al*[186] performed a 3D niche microarray on 1536-well plates that could control five characteristics: Matrix mechanical properties, ECM proteins, cell–cell interaction proteins, soluble factors and proteolytic degradability (matrix metalloproteinase sensitivity). Researchers cross-linked branched PEG-based macromers with specific peptide sequences susceptible to cell-secreted matrix metalloproteinases[186]. Then, they encapsulated mESCs in 3D PEG gels to investigate mESC proliferation and self-renewal properties in different combinations of microenvironments. From the completed studies, we can learn that HTS platforms, especially 3D platforms, are tools with great potential for constructing microenvironments to discover combinations of elements that could well facilitate *in vitro* neurogenesis, such as NSC proliferation and differentiation, and even the internal signaling in charge of those cell behaviors.

APPLICATIONS OF INDUCED NEURONAL CELLS

Since HTS technology can identify small molecules, specific genes and physiological microenvironments that contribute to neurogenesis *in vitro*, it is also important to focus on the clinical backgrounds and applications of the generated functional neurons or neural lineage cells using this technology.

Over the past 20 years, stem cell therapy and regenerative medicine have received considerable attention and have been expected to be applied in the treatment of CNS diseases, especially neurodegenerative diseases. The potential of stem cell therapy for CNS disease treatment lies in the capacity of

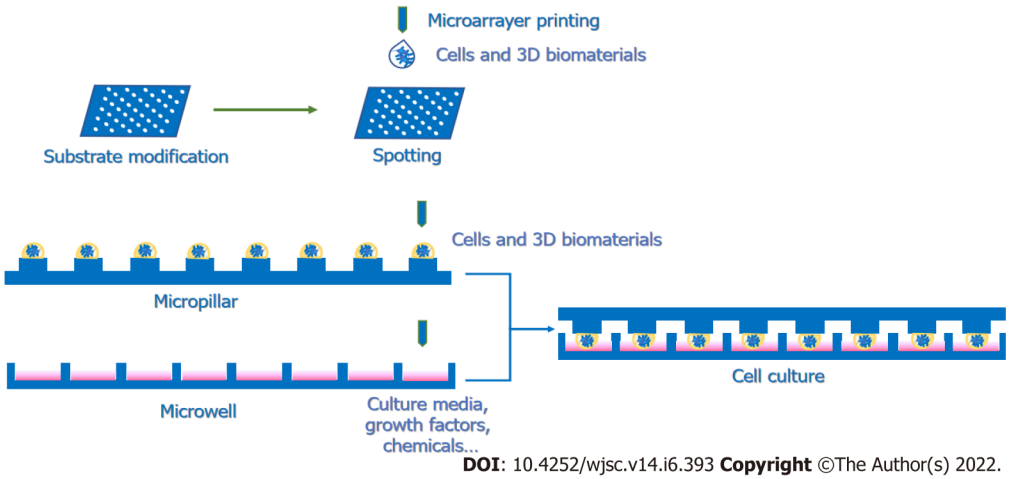

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Figure 2 Procedures for 3D microenvironment screening. The mixture of neural cells and 3D biomaterials is printed through a microarrayer and subsequently spotted on the substrate of microarrays or micropillars. Then, culture medium is added to the microarray, and the micropillar is stamped into the microwell containing culture medium, including various combinations of growth factors and chemicals.

NSCs to compensate for lost neurons with differentiated functional neurons, rebuild neural networks, secrete neurotrophic factors and reduce neuroinflammation to increase the survival rates of transplanted cells and healthy neurons[187,188]. Before clinical trials, studies of stem cell transplantation were conducted on animal disease models to examine the safety and efficiency of stem cell therapy. Recently, various studies have been carried out using primary NS/PCs, neural precursors, immortalized neural stem cell lines, iPSC-derived NS/PCs, and directly reprogrammed neural precursor cells to treat spinal cord injury (SCI)[189,190], AD[191,192], PD[193-195], Huntington's disease (HD)[196,197] and ALS[198] in animal models, and the symptoms of those diseases were relieved to some extent. For instance, the long-term selective stimulation of hM3Dq-expressing human iPSCs (hiPSCs)-derived NS/PCs enhanced their BDNF secretion, neuron-to-neuron interactions and synaptic activity in the surrounding host tissue in mouse SCI models, suggesting that enhancing neural activity and interactions with other cells in the neural circuit can be an effective way to improve the therapeutic effect of stem cell transplantation[199]. Furthermore, the cografting of MSCs and dopaminergic precursors at the appropriate dose can help to enhance the survival of dopaminergic neurons in PD rat models[200]. To better improve the survival, proliferation, differentiation and integrity of transplanted stem cells, growth factors[201,202], neurotrophic factors[203-206], hormones[207] and pharmacological treatments[208-211] have been applied with stem cells as methods of combination therapy. Additionally, genetic modification of engrafted stem cells can help to maintain long-term persistence, integrate into host tissues, and facilitate differentiation and maturation. The overexpression of growth factors[212], neurotrophic factors[213-218], morphogens[219] and transcription factors[220] in NS/NPCs is a widely applied method to further improve the therapeutic effect on CNS diseases and injuries.

For clinical studies, NPCs were transplanted into the dorsal putamina of patients with moderate PD, and a four-year evaluation was performed, which found this transplantation surgery to be safe and lacking in immune response or adverse effects[221]. Motor improvement and enhanced midbrain dopaminergic activity were shown, although they decreased somewhat over four years[221]. A long-term phase I clinical trial also proved the safety of hNSCs and found a transitory decrease in the progression of the ALS Functional Rating Scale Revised up to four months post-transplantation[222]. These results are promising, and in future studies, neural precursors derived from iPSCs or somatic cells, especially fibroblasts, can be applied to clinical studies to show safety and efficiency. For an allogeneic approach, developing an iPSC bank based on the human leukocyte antigen haplotype could provide more possibilities for stem cell therapy[223], and the genetic editing of patient-derived iPSCs is also a feasible approach for autologous stem cell transplantation[224]. Meanwhile, exploring other cell sources for stem cell therapy could help broaden the field for clinical studies. Recently, a research group used NSCs isolated from midbrain organoids, which are generated from hPSCs, and transplanted them into rat PD models[225]. The results showed midbrain dopaminergic neuron engraftment and reproducible behavioral restoration in those PD models[225].

Neural lineage cells generated *in vitro* are also cell sources for neural tissue engineering, as they are becoming an attractive option for CNS disease treatment, considering that the support of 3D scaffolds can mimic the microenvironments that help the engrafted cells survive, integrate and differentiate. For brain injury repair, the injection of NSCs with a hyaluronate collagen scaffold loaded with controlled release of bFGF can recover cognitive function through the promotion of survival, differentiation and synaptic formation of NSCs in traumatic brain injury (TBI) rats[226]. Chitosan scaffolds are also common options for the neural tissue engineering treatment of TBI in animal models[227-229]. In

addition to hydrogel materials, researchers also use porous scaffolds to prevent the collapse of scaffolds and provide enough space for neural differentiation, metabolic exchange, and neurite extension of grafted cells[230,231]. For SCI repair, collagen microchannel scaffolds and gelatin sponge scaffolds carrying NSCs with drugs or neurotrophic factors have enhanced tissue repair efficiency in SCI animal models[232,233]. Importantly, with the capacity to construct complex 3D microstructures, 3D bioprinting techniques have been applied to create CNS architectures for regenerative medicine. For instance, a microscale continuous projection printing method can print 3D hydrogel scaffolds within 1.6 s to fit the size of injured spinal cords in rodent models; this technology is also scalable to human size [234]. The 3D-printed neural tissues loaded with NS/PCs can integrate into host tissues, promote axon regeneration, and improve spinal cord functions in animal models[234,235]. In clinical trials, the collagen scaffold (NeuroRegen Scaffold; NRS) has been used with MSCs to treat SCI patients, and the recovery of sensory and motor functions was observed[236]. Another study transplanted NRS loaded with autologous bone marrow mononuclear cells into SCI patients, and in some patients, partial shallow sensory and autonomic nervous functional improvements could be observed, but the recovery of motor functions was not observed[237]. Therefore, with the development of various 3D scaffolds and 3D bioprinting techniques, neural tissue engineering has great potential to contribute to CNS disease treatment in further studies.

Functional neurons can also be used for *in vitro* modeling to achieve a better comprehension of CNS diseases and neural development mechanisms. For neural disease modeling, AD models have been constructed utilizing hNPCs with familial AD (FAD) genes in Matrigel-based 3D culture systems, and aggregated p-tau proteins and amyloid-β deposits resembling AD pathology were observed[22]. Furthermore, they showed that a high amyloid-β42/40 ratio could drive Aβ accumulation and phosphorylated tau protein accumulation in this 3D AD model[238]. To recapitulate neuroinflammation in AD, they also conducted a 3D AD triculture model containing hNPC-derived AD neurons/astrocytes and subsequently plated microglia in the microfluidic platform[239]. The results showed that migrating microglia, the upregulation of AD-related proinflammatory factors and the toxic effects of microglia on neurons and astrocytes could be observed in the 3D AD triculture model[239]. In addition to AD models, PD models have also been established using 3D culture with *in vitro* neural differentiation. Taylor-Whiteley *et al*[240] first constructed a 3D PD model by differentiating human SH-SY5Y neuroblastoma cells into dopaminergic cells with RA and BDNF cultured in Matrigel[240]. Next, they treated cells with preformed a-synuclein (a-syn) oligomers and observed a-syn-positive inclusions that resemble *in vivo* Lewy bodies in morphology[240]. Organoids have also been proven to be effective tools for 3D modeling. Kim *et al*[241] utilized hiPSCs with leucine-rich repeat kinase 2 G2019S mutation, which is a well-known trigger of late-onset familial and sporadic PD, to generate 3D midbrain organoids. From the 3D organoid model, they identified the TXNIP gene, which can contribute to the generation of α-syn in LRRK2-associated PD[241]. Another research group built a 3D sporadic AD model by treating brain organoids with human serum to mimic the serum exposure caused by a blood–brain barrier breakdown in AD[242]. AD-like pathologies could be observed in serum-exposed brain organoids, with increases in Aβ aggregates, phosphorylated microtubule-associated tau protein (p-Tau) levels, synaptic loss, apoptosis, and impaired neural networks[242]. In addition, 3D models aimed at other neural system diseases, including HD[243], hypoxic brain injury of prematurity[244] and brain tumors[245], have also been established. These 3D disease models provide us with feasible and valid platforms for future studies of disease pathogenesis and drug screening.

In addition to disease modeling, *in vitro* 3D models can also be constructed to recapitulate neural development. To study neural tube morphogenesis *in vitro* through 3D culture, Ranga *et al*[23] first performed combinational HTS to screen out appropriate parameters of 3D matrices, based on which they investigated the effects of early developmental signaling molecules, including RA, Shh, Wnt-3a, BMP4 and FGF8, on dorsal-ventral (D-V) patterning with their 3D neural tube model. Another study cultured mESCs in Matrigel or defined 3D scaffolds containing laminin and entactin or PEG and induced floor plate formation and D-V pattering with RA[246]. Mariani *et al*[247] induced human iPSCs to serum-free, floating embryoid body-like, quick aggregates with embryonic dorsal telencephalon properties, which could be used as an *in vitro* 3D model for human cortical development. With the use of cerebral organoids, gene expression programs and epigenetic signatures during human brain development were recapitulated, as well as the interaction patterns between different brain regions[248-250]. These models mimicking neural development could be applied to explore mechanisms underlying organogenesis and cell–cell interactions during neurogenesis[66,251] and could also be an option for studying neural genetic disorders[252], as well as a platform for drug screening.

LIMITATIONS AND PROSPECTS

Improving the conversion efficiency using small molecules and activating endogenous loci

Conversion efficiency is often discussed in articles focusing on cellular reprogramming and neural differentiation, which refers to the ratio of the target cell types to the initial cell types. The improvement of neural conversion efficiency is an important subject to address to increase the purity and efficiency of

generated neurons for future clinical use. To overcome these difficulties, small molecules have been screened out to replace transcription factors, as small molecules can improve the conversion efficiency of cellular reprogramming compared to the overexpression of transcription factors[253]. For instance, VPA can enhance the reprogramming efficiency of somatic cells to iPSCs[118,121], and CHIR99021, LDN193189, and A83-01 can further improve the neural induction rate[136]. Significantly, the application of CRISPRa is capable of greatly enhancing reprogramming efficiency by targeting the human embryo genome activation-enriched Alu motif, leading to more efficient activation of Nanog and Rex1[254]. Therefore, activating endogenous loci controlling cellular reprogramming and neural lineage induction can be an effective way to increase neural conversion efficiency. An alternative way is to selectively ablate proliferative cells and keep functional neurons for the sake of guaranteeing the safety and efficiency of stem cell transplantation. This research was performed *via* pharmacological activation of the suicide gene within weeks after transplantation, and the yield of dopaminergic neurons and the recovery of motor functions were not affected by diminishing the graft size in the PD rat model [255]. Thus, in future studies, HTS technology can still play an important role in screening small molecules and endogenous genes, which can aid in improving the conversion efficiency and generate more functional neurons.

Developing an organoid/spheroid-based HTS system

Over the decades, testing probes of HTS methods have been developed from molecules to cells and even to tissues/microenvironments and organoids. Currently, molecular, cell- and tissue-based screening systems have come into use, and it is also quite important to investigate organoid-based HTS devices with increased producibility and reduced heterogeneity between batches, allowing for large-scale screening[256]. Jorfi *et al*[257] screened FAD-mutated hNSCs or iPSC-derived neurospheroids with a 96-well cell culture plate with 1536 microwells. They embedded the neurospheroids in Matrigel and screened several chemical compounds to assess their impact on neural differentiation[257]. The establishment of a high-throughput bioengineered human cardiac organoid in the 96-well format was also reported[258]. After that, 105 hit compounds from approximately 5000 candidates, which were screened from iPSC-derived cardiomyocytes in the 2D platform, were screened over a 3-log scale concentration range that requires approximately 1000 human cardiac organoids to develop compounds with the capacity for cardiomyocyte proliferation[259]. Another study generated kidney organoids from hiPSCs utilizing multiwell plates, and this HTS-compatible platform was used to screen out an inhibitor of nonmuscle myosin II ATPase activity as a specific activator of polycystic kidney disease cystogenesis in organoids[260]. Renner *et al*[261] also developed an automated workflow that could integrate midbrain organoid culture, immunostaining and high-content imaging for high-throughput chemical screening using a 96-well format, which could save manual operation and improve the compatibility of organoid culture and HTS. Although high-content imaging analysis has been a powerful tool to evaluate organoid generation, for brain organoids, it is probable that the evaluation of neural circuit dynamics, such as that through 3D microelectrode arrays, could become a standard in upcoming studies [262,263]. Furthermore, combinations with the automated workflow of organoid culture and artificial intelligence can shed light on CNS disease modeling and drug discovery for clinical trials[264].

Improving microfluidic-based HTS systems

Over the years, HTS devices have been developed, ranging from multiwell plates to microarrays; notably, microfluidic devices are gradually showing their features in HTS technology. The lab-on-chip method has contributed to this development. Schudel *et al*[265] designed a microfluidic chip to separate cell clusters by dividing the chip into one part for siRNA patterning and another for target screening to study virus–host interactions. Furthermore, to improve the screening efficiency after cell transduction, Wang *et al*[266] designed a droplet-based microfluidic platform compatible with single-cell screening to identify the yeast Saccharomyces cerevisiae with elevated protein production through RNAi screening and searched for genetic targets capable of improving protein secretion. Han *et al*[267] first utilized a CRISPR/Cas9 screen on a microfluidic platform, also called a microfluidic separation chip, on which cells transduced with the lenti-CRISPR kinase library were sorted to examine transport distances to evaluate cell deformability[267]. For chemical screening, Titmarsh *et al*[268] constructed a high-density microbioreactor array that could provide 8100 chambers for the proliferation of hPSCs or hPSC-derived cardiomyocytes. They found that CHIR99021 showed the best effect on human cardiomyocyte proliferation among purmorphamine, IGF-1 and FGF-2[268]. Although the microfluidic array could provide thousands of chambers as reactors, the numbers of candidates allowed for one screening are usually limited. Thus, exploiting microfluidic devices that are able to hold more isolated channels for screening more candidates at a time has great potential.

However, the HTS platforms currently available for cell/organoid-based screening are mainly well plates that lack automation and integration and commonly cause reagent waste. Therefore, in further research, the microfluidic platform shows great promise to achieve a higher throughput and autocontrolled and integrated properties. For instance, Schuster *et al*[269] designed an automated microfluidic 3D cellular and organoid culture platform for the culture of pancreatic ductal adenocarcinoma organoids generated from single cells from patients. The platform could contain 20 independent experimental conditions and 200 individual chambers that are large enough to hold growing organoids[269].

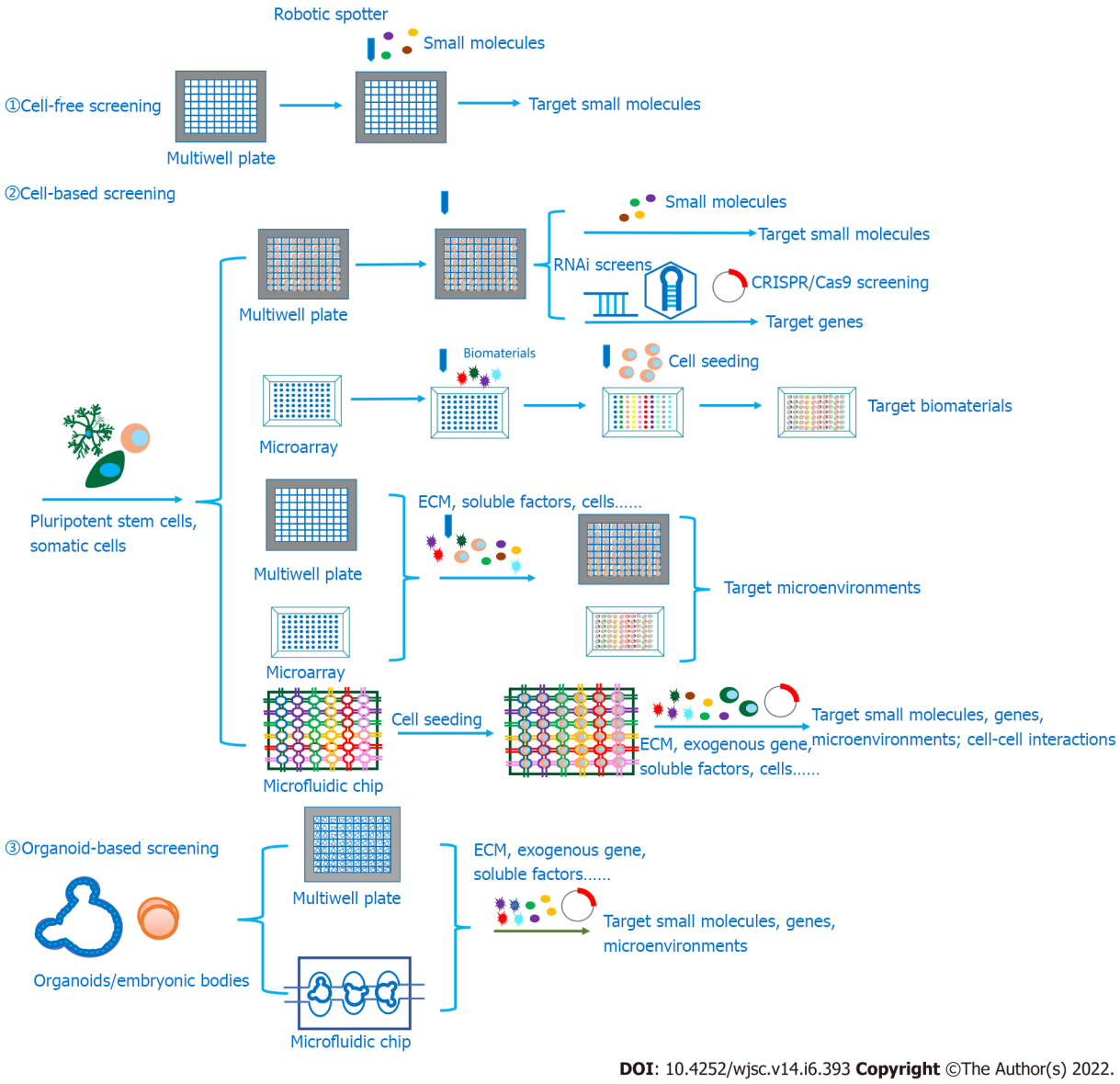

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Figure 3 Summary of high-throughput screening platforms and their functions. Cell-free, cell-based and organoid-based HTS platforms can screen out target small molecules, genes, biomaterials and microenvironments to promote the survival, proliferation, differentiation and maturation of pluripotent stem cells, induce the transdifferentiation of somatic cells to other cell types, and even generate specific organoids for further applications, including disease modeling, development modeling, drug screening, tissue engineering and regenerative medicine. High-throughput screening (HTS) platforms include multiwell plates, microarrays and microfluidic chips, indicating the great potential in HTS in future studies. HTS: High-throughput screening; ECM: Extracellular matrix.

The researchers performed dynamic and combinational drug screening and recorded the incidences of cellular apoptosis and death to evaluate the treatment effect of the temporal drug combinations.

In addition, microfluidic devices could also be utilized to perform cell coculture using droplet-based microfluidic systems, which could function in studying the microenvironments of cell–cell interactions under high-throughput conditions[270]. Other researchers have also designed high-throughput 3D coculture systems on microfluidic chips[271,272]. With these methods, HTS could be performed on these platforms to screen out 3D microenvironments containing cellular interactions, such as synaptic connections between neurons and astrocytes. In addition, microfluidic chips have been applied in generating concentration gradients of biomolecules to study steepness-dependent neural chemotaxis on high-throughput 3D platforms[273]. Rifes *et al*[274] constructed a microfluidic platform to generate gradients of CHIR99021 to activate Wnt signaling, and they modeled neural tube development in this 3D microfluidic system. Therefore, in future studies, HTS will be performed on microfluidic systems due to their capacity to better recapitulate the microenvironments *in vivo*, which is a strategy that shows great promise.

CONCLUSION

HTS technologies are playing increasingly important roles in neurogenesis *in vitro* due to their ability to screen out crucial genes controlling neural lineage determination, small chemical molecules regulating cell fate, and microenvironments, including 3D matrices, soluble factors, physical parameters and interactions with other cell types (Figure 3). After screening out suitable microenvironments, these culture conditions could be applied in generating mature and functional neurons, neural tissues and organoids *in vitro* for further applications, such as 3D modeling and drug screening, to investigate neural diseases or developmental mechanisms and explore medical solutions. With the requirements of 3D models, 3D-based screening with tissues or organoids is developing to better evaluate screening outcomes from an overall perspective than molecular or cell-based screening can. Meanwhile, the screening devices are trending toward minimization, automation and integration, from multiwell plates to microarrays and microfluidic devices, to conduct the screening process in a high-throughput manner that requires less time and consumes fewer reagents. Today, the need for combinational screening is growing, as investigations of the interactions between different drugs or environmental factors are vital to developing combined therapies and novel culture conditions. In addition, it is notable that microfluidics makes it easier to perform high-throughput combinational screening with nanodroplets and microwell array plates that can hold only two nanodroplets in a well[275]. Overall, from past studies and due to the fast development of HTS devices, we anticipate that HTS technologies will be able to make great contributions to *in vitro* neurogenesis and solve other problems in regenerative medicine in future studies.

To conclude, HTS technology could help to dissect the mechanisms of genetic regulation during neurodevelopment, identify niche-targeted small molecules and secreted factors to promote endogenous NSC activation for clinical treatment, and screen out biomaterials and other microenvironment elements to generate more functional and mature neurons with specific subtypes and improved purity, which could be used to establish 3D neural disease or developmental models. Although some 3D *in vitro* microenvironments cannot be reconstructed based on HTS at the present time, we still predict that HTS will be a promising tool for defining microenvironments for higher efficiency modelling.

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META-ANALYSIS

Role of stem cells-based in facial nerve reanimation: A meta-analysis of histological and neurophysiological outcomes

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Abstract

BACKGROUND

Treatments involving stem cell (SC) usage represent novel and potentially interesting alternatives in facial nerve reanimation. Current literature includes the use of SC in animal model studies to promote graft survival by enhancing nerve fiber growth, spreading, myelinization, in addition to limiting fibrotic degeneration after surgery. However, the effectiveness of the clinical use of SC in facial nerve reanimation has not been clarified yet.

AIM

To investigate the histological, neurophysiological, and functional outcomes in facial reanimation using SC, compared to autograft.

METHODS
Our study is a systematic review of the literature, consistently conducted according to the preferred reporting items for systematic reviews and meta-analyses statement guidelines. The review question was: In facial nerve reanimation on rats, has the use of stem cells revealed as effective when compared to autograft, in terms of histological, neurophysiological, and functional outcomes? Random-effect meta-analysis was conducted on histological and neurophysiological data from the included comparative studies.

RESULTS
After screening 148 manuscript, five papers were included in our study. 43 subjects were included in the SC group, while 40 in the autograft group. The meta-analysis showed no significative differences between the two groups in terms of myelin thickness [CI: -0.10 (-0.20, 0.00); I^2 = 29%; P = 0.06], nerve fibers diameter [CI: 0.72 (-0.93, 3.36); I^2 = 72%; P = 0.6], compound muscle action potential amplitude [CI: 1.59 (0.59, 3.77); I^2 = 89%; P = 0.15] and latency [CI: 0.66 (-1.01, 2.32); I^2 = 67%; P = 0.44]. The mean axonal diameter was higher in the autograft group [CI: 0.94 (0.60, 1.27); I^2 = 0%; $P \leq 0.001$].

CONCLUSION
The role of stem cells in facial reanimation is still relatively poorly studied, in animal models, and available results should not discourage their use in future studies on human subjects.

Key Words: Facial nerve; Palsy; Reanimation; Coaptation; Stem cells; Nerve fibers; Functional outcome

Core Tip: Our meta-analysis of studies comparing the use of autograft and stem cells for facial nerve reanimation in rats suggest that there appears to be no advantages in favor of stem cells, according to the evaluated histological and neurophysiological outcomes. Stem cell treatments have proven to be an interesting and viable option in numerous fields of surgery that have vast supporting scientific and clinically applicable literature. The role of stem cells in facial reanimation is still relatively new and poorly studied due to the liming nature and number of studies carried out exclusively in animal models.

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INTRODUCTION

Facial nerve (FN) palsy (FNP) represents a relevant issue, which poses a great burden on socio-economical health-related costs[1]. This condition constitutes a limitation in social relations, eventually affecting psycho-mental health[2]. Other than facial movements limitations, a severe FN disfunction results in functional disorders such as ipsilateral corneal ulcerations and unvoluntary drooling[3]. There are several medical specialists that need to be involved in the management of these patients, which include neurologists, neurosurgeons, ophthalmologists, maxillo-facial surgeons, ENTs, psychiatrics, and physiotherapists. A multidisciplinary management from numerous specialists tends to make this topic of wide interest with a large audience of readers, including different medical and paramedical fields[4,5].

Traumatic injuries, infectious diseases, metabolic disorders, and iatrogenic causes may determine different grades of FNP, requiring specific treatments according to the single case specifics[4]. In the short-to-midterm facial palsy, conservative management is usually preferred in cases of facial nerve anatomical preservation, while reconstructive techniques, such as nerve grafting, or flap harvesting are considered in patients showing facial nerve interruption or non-spontaneous restoration for longer than 6 mo[4]. The functional-aesthetic outcome, however, is often lower than expected after reconstructive surgeries. In addition, cranial nerves need to be partially sacrificed for the proximal coaptation of the nerve graft.

A current frontier in facial nerve reanimation are potentially represented by stem cells (SC). The role of SC in facilitating and accelerating nerve fibers spreading throughout grafts, ameliorating the myelinization, and reducing fibrotic degeneration have been recently reported in animal models[6-9]. The aim

of our systematic review of the literature and meta-analysis of the comparative studies available in current literature was to investigate the histological, neurophysiological, and functional outcomes in facial reanimation using SC, compared to autograft.

MATERIALS AND METHODS

Study design

The present study is a systematic review of the literature, consistently conducted according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement.

Review question

The review questions, according to the PRISMA statement, was formulated following the PICO (P: patients; I: intervention; C: comparison; O: outcomes) scheme, as it follows: In facial nerve reanimation on rats (P), has the use of stem cells (I) revealed as effective when compared to autograft (C), in terms of histological, neurophysiological, and functional outcomes (O)?

Inclusion and exclusion criteria

Screened papers were considered for eligibility if they: focused on the use of SC for FN reanimation in rats; included a comparative group with autograft; reported the type of SC, histological analysis of myelinization of nerve fibers, neurophysiological analysis of the compound muscle action potential (CMAP) amplitude and latency, data on the residual mobility, and the length of the follow-up (FU). Exclusion criteria included language other than English, non-comparative studies, and non-reported quantitative data for analysis. Papers reporting incomplete or not pool- able data, such as means missing of standard deviations or medians missing of interquartile ranges, were excluded or included only for the follow-up periods during which the data were complete.

Search strategy

Four different medical databases (PubMed, Scopus, Cochrane Library, Mendeley) were screened for identifying pertinent papers, using Reference Citation Analysis (https://www.referencecitation-analysis.com/). The search terms "stem cell", "facial nerve", "regeneration", "repair", "functional restoration", "reanimation" were combined using the Boolean operators "AND" and "OR". In the first review round, Title and Abstract of the papers were independently screened by two authors (R.P. and A.P.). Duplicated papers were excluded from the screening. In the second review round, papers included for the Full text analysis were screened, and considered for inclusion according to the inclusion criteria. The references of the included papers were then screened for papers erroneously missed in the first review round (forward search). Papers not considered as eligible were excluded with reason. Any discordance in the screening process were solved by consensus with a third senior author (L.R.). Included papers were considered for data analysis and evidence synthesis.

Outcome measurements

Title, list of authors, year and journal of publication were collected for every included paper. Animal type, number per each treatment group, surgical strategy, and the type of cells used were databased.

The following outcomes were extracted from the included papers: (1) Histological outcomes: myelin thickness, density of myelinated fibers, number of axons, axonal density, axonal diameter; (2) Neurophysiological outcomes: CMAP amplitude (mV), CMAP latency (ms), CMAP duration (ms); (3) Functional outcomes: residual mobility of the vibrissae; and (4) Complications.

Statistical analysis

Data of the study populations were summarized using proportion and weighed means. The mean and standard deviations in individual studies were estimated from the median and interquartile ranges, when needed, according to the method described by Wan *et al*[10]. Pooled mean differences for continuous variables were computed between outcome groups with a random effects model[11]. Comprehensive meta-analysis software (Review Manager - RevMan 5.4.1 The Cochrane Collaboration, 2020) was used for pooling data. *P*-value was considered significant at $\alpha < 0.05$.

RESULTS

Studies included in the analysis

The first round of search on the selected database identified 148 abstracts to be screened. According to our inclusion criteria, five papers met these criteria and were included in the final meta-analysis of comparative studies[12-16]. See Figure 1 - Search strategy.

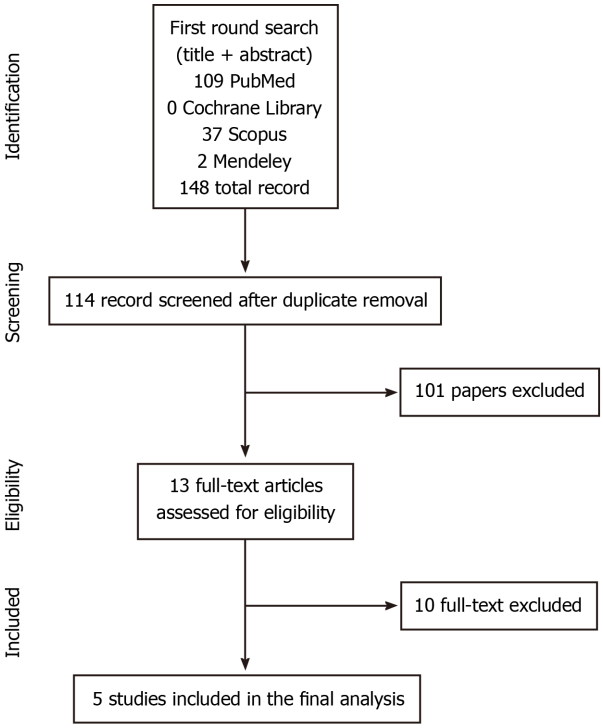

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Figure 1 Search strategy flowchart.

From the five included papers, 43 subject were included in the study group (SC), while 40 were included in the control group (Autograft). In the study group, adipose-derived stem cells (ASC) were used in 26 subject, stem cells from human exfoliated deciduous teeth (SHED) in 10, and bone marrow stem cells (BMSC) in 7.

Myelin thickness (μm)

The evaluation of myelin thickness was reported in three[12,13,15] of the five included studies, on a total of 28 subjects from the study group and 28 from the control group. The meta-analysis showed no significative differences in terms of myelin thickness between the two groups, and a low heterogeneity between the contributing studies [CI: 0.10 (-0.20, 0.00); I^2 = 29%; P = 0.06] (Figure 2A).

Nerve fibers diameter (μm)

The nerve fiber diameters were evaluated in two[12,15] of the five included studies, which included data of 16 subjects from the study group and 16 from the control group. Our data analysis showed no significative differences in terms of nerve fibers diameter between the two groups, and a high heterogeneity between the contributing studies [CI: 0.72 (-0.93, 3.36); I^2 = 72%; P = 0.6] (Figure 2B).

Axonal diameter (μm)

Two studies[12,16] reported data on the axonal diameter, including a total of 16 subjects in the study group and 16 in the control group. The pooled analysis showed a significatively higher axonal diameter in the control group, and a very low heterogeneity between the contributing studies [CI: 0.94 (0.60, 1.27); I^2 = 0%; P < 0.001] (Figure 2C).

CMAP amplitude (mA)

The CMAP amplitude was reported in three[12,15,16] of the five included studies, which was evaluated in 26 subjects from the study group and 23 from the control group. Our data analysis showed that no significative differences existed between the treatment groups, although a high heterogeneity was measured between the contributing studies [CI: 1.59 (0.59, 3.77); I^2 = 89%; P = 0.15] (Figure 2D).

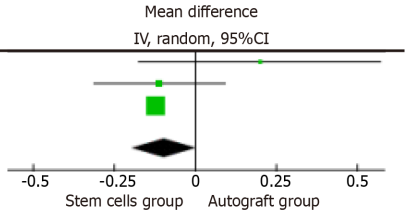
CMAP latency (ms)

Quantitative data on CMAP latency were reported in two[12,15] of the five included studies that included 16 subjects from the study group and 16 from the control group. The data analysis showed that no significative differences existed in terms of CMAP latency between the study and the control group, although a medium-to-high heterogeneity was calculated between the contributing studies [CI: 0.66 (-1.01, 2.32); I^2 = 67%; P = 0.44] (Figure 2E).

A

Study or subgroup	Stem cells Mean	SD	Total	Autograft Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Fujll *et al*	0.8	0.5	8	0.6	0.2	8	6.6%	0.20 [-0.17, 0.57]
Kamel *et al*	0.68	0.29	8	0.79	0.03	8	18.5%	-0.11 [-0.31, 0.09]
Sun *et al*	0.51	0.01	10	0.63	0.02	10	74.9%	-0.12 [-0.13, -0.11]
Total (95%CI)			26			26	100%	-0.10 [-0.20, 0.00]

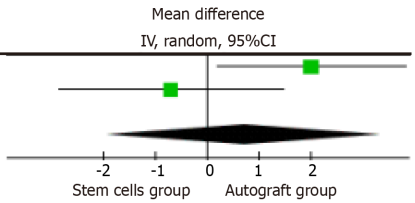
Heterogenelty: Tau^2 = 0.00; Chi^2 = 2.83, df = 2 (P = 0.24); I^2 = 29%
Test for overall effect: Z = 1.90 (P = 0.06)



B

Study or subgroup	Stem cells Mean	SD	Total	Autograft Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Fujll *et al*	5.7	2.2	8	3.7	1.4	8	52.5%	2.00 [0.19, 3.81]
Kamel *et al*	5	2.2	8	5.7	2.2	8	47.5%	-0.70 [-2.86, 1.46]
Total (95%CI)			16			16	100%	0.72 [-1.93, 3.36]

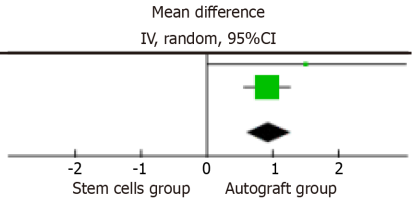
Heterogenelty: Tau^2 = 2.62; Chi^2 = 3.54, df = 1 (P = 0.06); I^2 = 72%
Test for overall effect: Z = 0.53 (P = 0.60)



C

Study or subgroup	Stem cells Mean	SD	Total	Autograft Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Fujll *et al*	4	1.8	8	2.5	1.2	8	5%	1.50 [0.00, 3.00]
Perelra *et al*	3.04	0.49	8	2.13	0.07	8	95%	0.91 [0.57, 1.25]
Total (95%CI)			16			16	100%	0.94 [0.60, 1.27]

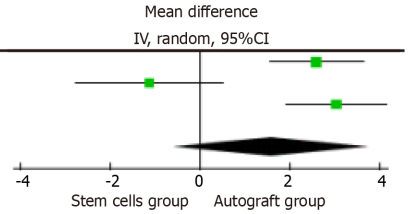
Heterogenelty: Tau^2 = 0.00; Chi^2 = 0.57, df = 1 (P = 0.45); I^2 = 0%
Test for overall effect: Z = 5.51 (P < 0.00001)



D

Study or subgroup	Stem cells Mean	SD	Total	Autograft Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Fujll *et al*	4.2	1.3	8	1.6	0.8	8	34.7%	2.60 [1.54, 3.66]
Kamel *et al*	3.222	1.779	8	4.352	1.587	8	31.0%	-1.13 [-2.78, 0.52]
Perelra *et al*	3.79	1.74	10	0.75	0.46	7	34.3%	3.04 [1.91, 4.17]
Total (95%CI)			26			23	100%	1.59 [-0.59, 3.77]

Heterogenelty: Tau^2 = 3.28; Chi^2 = 18.20, df = 2 (P = 0.0001); I^2 = 89%
Test for overall effect: Z = 1.43 (P = 0.15)



E

Study or subgroup	Stem cells Mean	SD	Total	Autograft Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Fujll *et al*	4.3	1	8	4.5	1.7	8	49.6%	-0.20 [-1.57, 1.17]
Kamel *et al*	4.3	1.7	8	2.8	0.9	8	50.4%	1.50 [0.17, 2.83]
Total (95%CI)			16			16	100%	0.66 [-1.01, 2.32]

Heterogenelty: Tau^2 = 0.97; Chi^2 = 3.05, df = 1 (P = 0.08); I^2 = 67%
Test for overall effect: Z = 0.77 (P = 0.44)

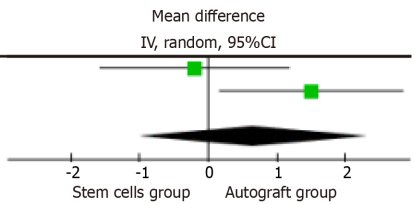


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Figure 2 Foster plots from the present meta-analysis. A: Myelin thickness; B: Nerve fibers diameter; C: Axonal diameter; D: Compound muscle action potential (CMAP) amplitude; E: CMAP latency. The Foster plots were generated using RevMan5.3 software. Influence of each included study in the pooled analysis is based on its weight, as specified in the dedicated column. The random effect model was used for data pooling. Heterogeneity is expressed as the I^2, and it is considered as low (< 25%), medium (< 50%), high (< 75%), or very high (> 75%), for a proper data interpretation.

Data on density of myelinated fibers, CMAP duration, and residual mobility was only reported in 1 of the 5 studies, thus preventing any data pooling for analysis.

DISCUSSION

Previous studies have reported data on the use of mesenchymal stem cells[6], dental pulp stem cells [17], gingiva-derived mesenchymal stem cells[18], bone-marrow derived stem cells, and adipose-tissue derived stem cells[7] in nerve regeneration after peripheral nerve injury. These treatments have demonstrated several advantages with regards to nerve fiber spreading, myelinization, and regeneration of the optimal perineural environment, thus tending to reduce fibrosis and inflammatory-

mediated disorders[7-9,18]. Adipose tissue has been used in peripheral nerve reconstruction after sciatic nerve transection in animal models, which seemed to provide relevant advantages in terms of nerve fiber density, axon area, and myelin area[19]. It is important, however, to demonstrate that histology translates to functional benefits, which was investigated by Schweizer *et al*[19] and Tuncel *et al*[20], using a swim test and a walking track analysis, respectively, thus confirming clinical effectiveness.

Standard reconstructive techniques use autograft for filling the gap between the proximal stump and the distal nerve in facial nerve reanimation[4]. Studies have shown that autograft use tend to be superior to acellular grafts in these cases, in which sensory nerves are considered as the gold standard graft for motor nerves reconstruction[8]. In designing our meta-analysis, we thus included only studies comparing autografts and stem cells for facial nerve reanimation in rats.

Our meta-analysis analysis of comparative studies highlighted that the use of BMSC, ASC, or SHED do not improve the histological and neurophysiological outcomes in facial nerve reanimation in rats in a short-term follow-up, compared to the use of autograft. Our analysis showed that the use of SC was able to slightly increase the mean myelin thickness, although the difference with the autograft group was not significative (P = 0.06). The mean axonal diameter, however, was significantly higher when using autografts (P < 0.001). It is important to note that the axons in the autograft groups were not distinguished in newer spreading axons and native fibers, thus need careful interpretation. Similarly, the mean nerve fibers diameter was not different between the study and control group (P = 0.06), although it was slightly wider in the autograft group.

With regards to the neurophysiological outcomes, our pooled analysis showed no differences between the study and control group in terms of CMAP amplitude (P = 0.15) and latency (P = 0.44). The innervated muscles did not seem to benefit from the use of stem cells for facial nerve reanimation, in terms of earlier and effective reinnervation. This should be carefully and critically interpretated, since the timing for reinnervation plays a relevant role in peripheral nerves surgery. The reinnervation itself may result as useless once the interested muscle has already experience a non-reversable degeneration once the long-term denervation occurred. It is important to note that the study protocols of the papers assessed in our study, set a maximum follow-up 13 wk, which may be too short and not sufficient in evaluating reinnervation after treatment. Nerve fibers regenerate 1mm per day in humans, which is assumed to be similar with regrowth rates in animals. Reinnervation may occur within up to 12 mo in patients undergoing surgery for facial reanimation[4]; therefore, data on reinnervation should be carefully interpreted, especially if based on short follow-up observation times. Limiting results with stem cells may be due to short healing time assessments as opposed to lack of efficacy. Only future studies based on extensive follow-up periods after treatment can provide true answers.

A critical analysis and discussion of our results are fundamental to provide a correct interpretation of our study. The histological findings and the non-reported significative differences between the use of autograft or stem cells could be of limited clinical use. The short follow-up time (of a maximum of 13 wk) may prove not to be sufficient in assessing medium-to-long term differences in new generated fibers spreading throughout the conduct. Furthermore, fibrotic degeneration may be influenced when using stem cells, and fibrosis, which normally occurs much later, may not have been an important factor in short time outcomes, yet of utmost importance in long term functional and histological results that could not be considered in these short follow-up studies.

The neurophysiological results showed no differences in terms of CMAP amplitude and latency. Once again, the short-term follow-up must be considered when evaluating the nerve conduction and the muscle activation. Accordingly, the similar neurophysiological outcomes between the two treatment groups cannot be considered as reliable for clinical application.

Functional outcomes in terms of residual movements and spontaneous movements restoration were not quantitatively reported, thus preventing any meta-data analysis. As reported in numerous papers, the functional outcomes should be considered as the primary outcome in facial reanimation techniques since it has a primary impact on the needs and satisfaction of the patient. The reinnervation and the histological pattern might play a marginal role in cases of non-functional restoration of muscles function.

Based on the results of our meta-analysis, the use of autograft should still be preferred in facial reinnervation, due to the non-significative differences compared to the use of stem cells. Current studies in literature based on animal subjects are limiting in terms of type of assessments, number of cases and short follow-up time evaluations, thus not sufficient in discouraging the clinical use of SC for facial reanimation.

Limitations

Our study has several limitations that need to be disclosed for a proper data interpretation. Only five studies only were included in the analysis; the type of rats was not the same throughout the studies; the surgical technique was not the same in the study protocols; different type of stem cells were used in each study; the follow-up time for outcomes evaluation was not homogeneous between the studies; there were no quantitative data on residual movements after treatment, thus preventing any analysis of functional outcomes.

CONCLUSION

Our meta-analysis of studies comparing the use of autograft and stem cells for facial nerve reanimation in rats suggest that there appears to be no advantages in favor of stem cells, according to the evaluated histological and neurophysiological outcomes. A higher heterogeneity amongst the included studies, short follow-up time periods and the limitations of our investigation should be carefully considered for a proper data interpretation. Stem cell treatments have proven to be an interesting and viable option in numerous fields of surgery that have vast supporting scientific and clinically applicable literature. The role of stem cells in facial reanimation is still relatively new and poorly studied due to the liming nature and number of studies carried out exclusively in animal models. Future studies based on longer follow-up with homogenous criteria, preferably on human subjects, can pave the way to stem cell therapy in patients with nerve palsy.

ARTICLE HIGHLIGHTS

Research background

Treatments involving stem cell (SC) usage represent novel and potentially interesting alternatives in facial nerve reanimation. Current literature includes the use of SC in animal model studies to promote graft survival by enhancing nerve fiber growth, spreading, myelinization, in addition to limiting fibrotic degeneration after surgery. However, the effectiveness of the clinical use of SC in facial nerve reanimation has not been clarified yet.

Research motivation

To investigate the histological, neurophysiological, and functional outcomes in facial reanimation using SC, compared to autograft.

Research objectives

The objectives of our systematic review of the literature and meta-analysis of the comparative studies available in current literature was to investigate the histological, neurophysiological, and functional outcomes in facial reanimation using SC, compared to autograft.

Research methods

Our study is a systematic review of the literature, consistently conducted according to the preferred reporting items for systematic reviews and meta-analyses statement guidelines. The review question was: In facial nerve reanimation on rats, has the use of stem cells revealed as effective when compared to autograft, in terms of histological, neurophysiological, and functional outcomes? Random-effect meta-analysis was conducted on histological and neurophysiological data from the included comparative studies.

Research results

After screening 148 manuscript, five papers were included in our study. 43 subjects were included in the SC group, while 40 in the autograft group. The meta-analysis showed no significative differences between the two groups in terms of myelin thickness [CI: -0.10 (-0.20, 0.00); I^2 = 29%; P = 0.06], nerve fibers diameter [CI: 0.72 (-0.93, 3.36); I^2 = 72%; P = 0.6], Compound Muscle Action Potential amplitude [CI: 1.59 (0.59, 3.77); I^2 = 89%; P = 0.15] and latency [CI: 0.66 (-1.01, 2.32); I^2 = 67%; P = 0.44]. The mean axonal diameter was higher in the autograft group [CI: 0.94 (0.60, 1.27); I^2 = 0%; $P \leq 0.001$].

Research conclusions

The role of stem cells in facial reanimation is still relatively poorly studied, in animal models, and available results should not discourage their use in future studies on human subjects.

Research perspectives

The role of stem cells in facial reanimation is still relatively new and poorly studied due to the liming nature and number of studies carried out exclusively in animal models. Future studies based on longer follow-up with homogenous criteria, preferably on human subjects, can pave the way to stem cell therapy in patients with nerve palsy.

FOOTNOTES

Author contributions: Ricciardi L wrote the outline, did the research, wrote the paper, and provided the final approval of the version of the article; Pucci R assisted in the research and writing of the manuscript; Piazza A and

Miscusi M assisted in the editing and making critical revisions of the manuscript; Lofrese G assisted in data analysis and their discussion; Scerrati A assisted in the review process, papers selection and data collection; Montemurro N assisted in the data collection and analysis; Raco A assisted in the writing, editing and making critical revisions of the manuscript; Ius T assisted in the research and revisions of the manuscript; Zeppieri M assisted in the conception and design of the study, writing, outline, final approval of the version of the article to be published and completed the English and scientific editing.

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LETTER TO THE EDITOR

Long noncoding RNAs in mesenchymal stromal/stem cells osteogenic differentiation: Implications in osteoarthritis pathogenesis

Daniel Quintero, Hugo C Rodriguez, Anish G Potty, Dimitrios Kouroupis, Ashim Gupta

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Abstract

This letter focuses on a recently published article that provided an exceptional description of the effect of epigenetic modifications on gene expression patterns related to skeletal system remodeling. Specifically, it discusses a novel modality of epigenetic regulation, the long noncoding RNAs (lncRNAs), and provides evidence of their involvement in mesenchymal stromal/stem cells osteo-/adipogenic differentiation balance. Despite focus on lncRNAs, there is an emerging cross talk between lncRNAs and miRNAs interaction as a novel mechanism in the regulation of the function of the musculoskeletal system, by controlling bone homeostasis and bone regeneration, as well as the osteogenic differentiation of stem cells. Thus, we touched on some examples to demonstrate this interaction. In addition, we believe there is still much to discover from the effects of lncRNAs on progenitor and non-progenitor cell differentiation. We incorporated data from other published articles to review lncRNAs in normal progenitor cell osteogenic differentiation, determined lncRNAs involved in osteoarthritis pathogenesis in progenitor cells, and provided a review of lncRNAs in non-progenitor cells that are differentially regulated in osteoarthritis. In conclusion, we really enjoyed reading this article and with this information we hope to further our under-

standing of lncRNAs and mesenchymal stromal/stem cells regulation.

Key Words: Long noncoding RNAs; Epigenetics; Mesenchymal stromal/stem cells; Degenerative bone diseases; Osteoarthritis; Osteoporosis

Core Tip: This letter summarizes that long noncoding RNAs (lncRNAs) are involved in mesenchymal stromal/stem cells (MSCs) osteo-/adipo-genic differentiation balance. We added that the interaction between lncRNAs and miRNAs is strongly involved in the regulation of the function of the musculoskeletal system, by controlling bone homeostasis and bone regeneration, as well as the osteogenic differentiation of stem cells. Additionally, MSCs/progenitor cells lncRNAs are involved in osteogenic differentiation, osteoarthritis pathogenesis, and lncRNAs in non-progenitor cells are differentially regulated in osteoarthritis.

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TO THE EDITOR

We read with great interest the review article by Xia *et al*[1], titled "Epigenetic regulation by long noncoding RNAs in osteo-/adipo-genic differentiation of mesenchymal stromal cells and degenerative bone diseases". We believe the article provides an exceptional description of the effect of epigenetic modifications on gene expression patterns related to skeletal system remodeling. Specifically, it discusses a novel modality of epigenetic regulation, the long noncoding RNAs (lncRNAs), and provides evidence of their involvement in mesenchymal stromal/stem cells (MSCs) osteo-/adipo-genic differentiation balance. We agree with the authors' insight that lncRNAs are relevant to clinical practice as altered MSCs differentiation status can be implicated in the initiation/progression of various musculoskeletal pathologies such as osteoarthritis and osteoporosis. We do, however, have several clarifications we wish to provide.

In the introduction, MSCs are defined as "a heterogenous population of cells which include fibroblast, myofibroblast and progenitor cells"[1]. Even though this definition was previously introduced by International Society for Cell & Gene Therapy Mesenchymal Stromal Cell Committee[2], it can be misleading within the present article as authors evaluate the effect of lncRNAs on cells that possess differentiation capacity and not fully differentiated cells (such as fibroblasts). Instead, authors could introduce MSCs as mesenchymal stromal/stem cells are fibroblast-like cells capable of multilineage differentiation at least *in vitro* that possess strong paracrine and immunomodulatory properties *in vivo*. Additionally, even though MSCs are originated from a single cell population during embryogenesis, authors should acknowledge that MSCs show intrinsic propensities to osteo-/adipo-genic differentiation strongly related to their tissue of origin and functional MSC subset heterogeneity[3]. This may significantly affect the role of specific lncRNAs on the overall epigenetic regulation of MSCs differentiation.

In the present article authors have nicely presented the interactions between lncRNAs and epigenetic modifiers during osteo-/adipo-genic MSCs' differentiation. However, in recent years the crosstalk between lncRNAs and miRNAs interaction has emerged as a novel mechanism in the regulation of the function of the musculoskeletal system, by controlling bone homeostasis and bone regeneration, as well as the osteogenic differentiation of stem cells[4]. We totally acknowledge that the topic of the present article is not miRNAs, however authors could elaborate more on this significant interaction. For example, ANRIL lncRNA was correlated with increased MSCs osteogenic differentiation in the present article. According to recent studies, the molecular mechanism of ANRIL lncRNA effects is based on its direct binding to circulating miR-7a involved in activating the NFKB signaling pathway[5]. Other lncRNAs that exert their osteoinductive activities on progenitor cells *via* binding to miRNAs are MALAT1 and PGC1β-OT1[6,7]. Similarly, HOTAIR lncRNA *via* miR-17-5p interaction inhibits osteogenic differentiation in individuals with a traumatic osteonecrosis of the femoral head. This is in relation to a variable activation of SMAD7 which directly influences osteoblastic differentiation[8]. On this basis of lncRNAs and miRNAs interactions, it seems that H19 lncRNA is a major regulator of MSCs osteogenic differentiation. Specifically, H19 lncRNA act *via* three modes of action: (1) Up-regulate miR-

Table 1 Supplementary information to Figure 1 detailing source and mechanism of activity associated with modified long noncoding RNAs

Upregulated			Downregulated		
lncRNAs	**Function**	**Ref.**	**lncRNAs**	**Function**	**Ref.**
DANCR	Increased proliferation and chondrogenesis	Wang *et al* [12], 2020	XIST	Increased inflammation and apoptotic rate	Lian *et al* [13], 2020
MALAT1	Decreased rate of synovial fibroblast proliferation	Nanus *et al* [14], 2020	NR024118	Inflammation, apoptosis, and ROS elevation	Mei *et al* [15], 2019
THRIL	Upregulated inflammatory injury and apoptosis	Liu *et al*[16], 2019	HULC	Increased inflammation	Chu *et al* [17], 2019
LINC0051	Results in anti-proliferative actions	Zhang *et al* [18], 2020	lncRNA-ATB	Increased inflammation	Ying *et al* [19], 2019
			OIP5-AS1	Decreased cell proliferation and migration, decreased cell anti-inflammatory mediator secretion	Zhi *et al* [20], 2020

lncRNAs: Long noncoding RNAs.

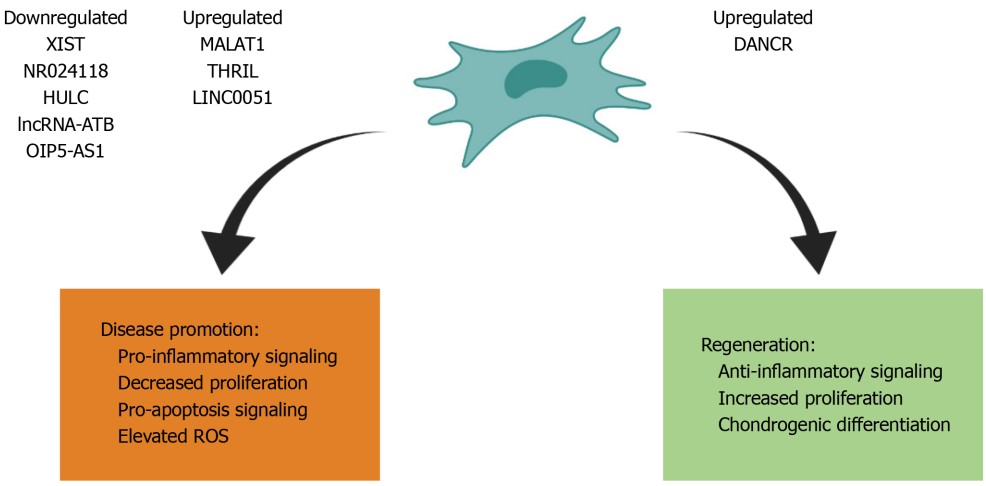

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Figure 1 Effects of various long noncoding RNAs on mesenchymal stromal/stem cells/progenitor cells for disease promotion and regeneration.

675 expression and inhibit the phosphorylation of TGF-β1 and Smad3; (2) inhibit the expression of miR-141 and miR-22 and promote Wnt/β-catenin signal transduction pathway; and (3) inhibit the expression of miR-107, miR-27b, miR-106b, miR-125a, and miR-17 resulting in Notch signaling pathway regulation [9-11].

Pathological mechanisms of osteoarthritis (OA) development involve the interplay of different OA symptoms, including inflammatory and degenerative changes that lead to destruction of articular cartilage, deranged chondrocyte regeneration, osteophyte formation, subchondral sclerosis and hyperplasia of synovial tissue. Yet, we must make a distinction between lncRNAs expression in progenitor cells and lncRNAs expression changes in terminally differentiated cells such as chondrocytes as their implication on cell differentiation and protein expression are remarkably different. Herein, in addition to the present article data we incorporated data from other literature to: (1) Review MSCs/progenitor cells lncRNAs involved in osteogenic differentiation; (2) determine MSCs/progenitor cells lncRNAs involved in OA pathogenesis; and (3) provide a review of lncRNAs in non-progenitor cells that are differentially regulated in OA.

On this basis, we identified four lncRNAs that are upregulated in MSCs/progenitor cells: DANCR, MALAT1, THRIL and LINC0051; and five lncRNAs are downregulated in MSCs/progenitor cells, specifically chondrogenic cell line ATDC5: XIST, NR024118, HULC, LncRNA-ATB, OIP5-AS1. A summary of these findings is featured in Figure 1 and Table 1[12-20].

lncRNAs strongly regulate chondrocytes expression patterns in both physiological and pathological conditions. Twelve different lncRNAs were upregulated in terminally differentiated chondrocytes. We summarize these findings in Figure 2 and Table 2[21-32].

Table 2 Supplementary information to Figure 2 detailing source and mechanism of activity associated with modified long noncoding RNAs

lncRNAs	Function	Ref.
ARFRP1	Increased apoptosis related proteins	Zhang *et al*[21], 2020
LOXL-1 AS1	Improved inflammation and proliferation rate	Chen *et al*[22], 2020
NEAT 1	Increases apoptosis, decreases autophagy, decreases viability	Liu *et al*[23], 2020
MFI2-AS1	Increases inflammation, ECM degradation, and apoptosis	Luo *et al*[24], 2020
PART1	Low cell proliferation and increased cellular apoptosis	Zhu *et al*[25], 2019
TNFSF10	Improves cellular proliferation, anti-apoptotic, and anti-inflammatory actions	Huang *et al*[26], 2019
XIST	Increases inflammation and apoptosis	Wang *et al*[27], 2019
FOXD2-AS1	Decreases inflammation, decreases ECM degradation	Wang *et al*[28], 2019
H19	Decreases proliferation, increases apoptosis, increases inflammation	Hu *et al*[29], 2019
SNHG16	Decreases proliferation	Fan *et al*[30], 2020
CTBP1-AS2	Decreases proliferation	Zhang *et al*[31], 2020
HOTAIR	Increases apoptosis	He *et al*[32], 2020

ECM: Extracellular matrix; lncRNAs: Long noncoding RNAs.

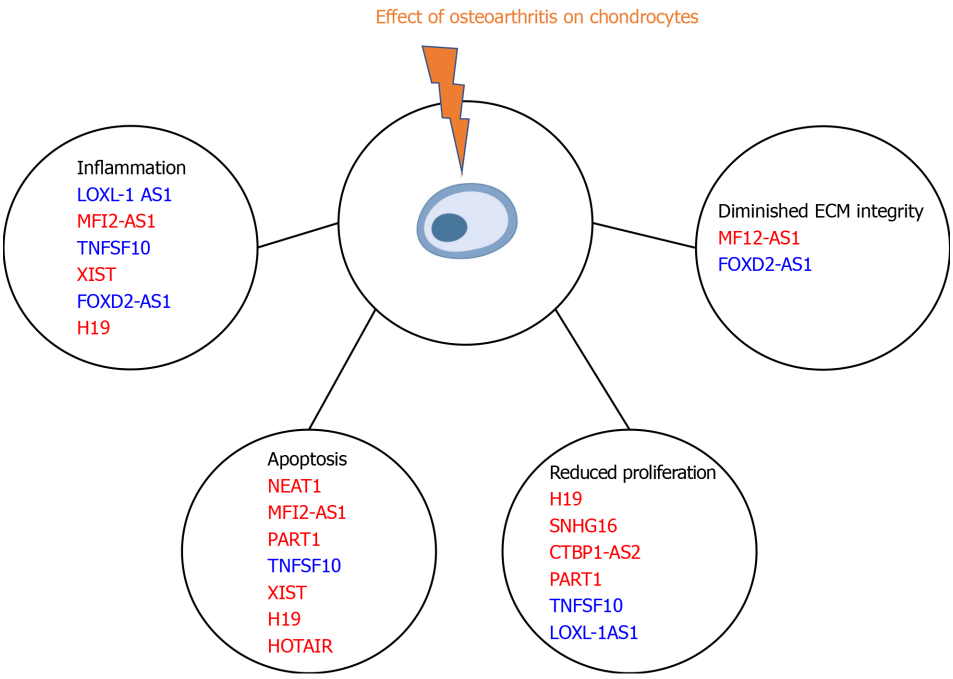

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Figure 2 Effects of various long noncoding RNAs on chondrocytes in osteoarthritis. Red text indicates promotion of pathogenesis, while blue text indicated regeneration by opposing pathogenic signaling. ECM: Extracellular matrix.

In conclusion, we believe there is still much to discover from the effects of lncRNAs on progenitor and non-progenitor cell differentiation. We incorporated data from a recent review article by Ghafouri-Fard *et al*[33] among other articles to: (1) Review lncRNAs in normal progenitor cell osteogenic differentiation; (2) determine lncRNAs involved in OA pathogenesis in progenitor cells; and (3) provide a review of lncRNAs in non-progenitor cells that are differentially regulated in OA. We provided a superficial review of lncRNAs expression and osteoarthritis to clarify what was mentioned and separated the regulation in progenitor and non-progenitor cells, which was not previously published. Again, we really enjoyed the reading by Xia *et al*[1] and with this information we hope to further our understanding of lncRNAs and mesenchymal stromal/stem cells regulation.

FOOTNOTES

Author contributions: Gupta A and Kouroupis D conceptualized the study; Quintero D, Rodriguez HC, Potty AG, Kouroupis D, and Gupta A outlined and designed the manuscript; Quintero D, Rodriguez HC, Kouroupis D and Gupta A drafted the manuscript; Potty AG, Kouroupis D and Gupta A critically reviewed and edited the manuscript; all authors approved the final version of the article for publication.

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