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**REVIEW**

- 406** Defining lung cancer stem cells exosomal payload of miRNAs in clinical perspective
Aramini B, Masciale V, Haider KH
- 422** Stem cell-based approaches: Possible route to hearing restoration?
Durán-Alonso MB
- 438** Recent advances of single-cell RNA sequencing technology in mesenchymal stem cell research
Zheng G, Xie ZY, Wang P, Wu YF, Shen HY
- 448** Energy metabolism in cancer stem cells
Zhu X, Chen HH, Gao CY, Zhang XX, Jiang JX, Zhang Y, Fang J, Zhao F, Chen ZG
- 462** Human hair follicle-derived mesenchymal stem cells: Isolation, expansion, and differentiation
Wang B, Liu XM, Liu ZN, Wang Y, Han X, Lian AB, Mu Y, Jin MH, Liu JY

MINIREVIEWS

- 471** Stem cell therapy for COVID-19 and other respiratory diseases: Global trends of clinical trials
Ji HL, Liu C, Zhao RZ
- 481** Multifaceted p21 in carcinogenesis, stemness of tumor and tumor therapy
Xiao BD, Zhao YJ, Jia XY, Wu J, Wang YG, Huang F
- 488** Strategies for treating oesophageal diseases with stem cells
Gao Y, Jin SZ

ORIGINAL ARTICLE**Basic Study**

- 500** Cytotoxicity of nonylphenol on spermatogonial stem cells *via* phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin pathway
Lei JH, Yan W, Luo CH, Guo YM, Zhang YY, Wang XH, Su XJ

Retrospective Study

- 514** High tibial osteotomy with human umbilical cord blood-derived mesenchymal stem cells implantation for knee cartilage regeneration
Song JS, Hong KT, Kong CG, Kim NM, Jung JY, Park HS, Kim YJ, Chang KB, Kim SJ

ABOUT COVER

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Defining lung cancer stem cells exosomal payload of miRNAs in clinical perspective

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Abstract

Since the first publication regarding the existence of stem cells in cancer [cancer stem cells (CSCs)] in 1994, many studies have been published providing in-depth information about their biology and function. This research has paved the way in terms of appreciating the role of CSCs in tumour aggressiveness, progression, recurrence and resistance to cancer therapy. Targeting CSCs for cancer therapy has still not progressed to a sufficient degree, particularly in terms of exploring the mechanism of dynamic interconversion between CSCs and non-CSCs. Besides the CSC scenario, the problem of cancer dissemination has been analyzed in-depth with the identification and isolation of microRNAs (miRs), which are now considered to be compelling molecular markers in the diagnosis and prognosis of tumours in general and specifically in patients with non-small cell lung cancer. Paracrine release of miRs *via* "exosomes" (small membrane vesicles (30-100 nm), the derivation of which lies in the luminal membranes of multi-vesicular bodies) released by fusion with the cell membrane is gaining popularity. Whether exosomes play a significant role in maintaining a dynamic equilibrium state between CSCs and non-CSCs and their mechanism of activity is as yet unknown. Future studies on CSC-related exosomes will provide new perspectives for precision-targeted treatment strategies.

Key words: Cancer; Cancer stem cells; Exosomes; Lungs; miRNA; Microvesicles; Non-small cell lung cancer

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Core tip: The role of cancer stem cells (CSCs) in tumour aggressiveness, progression, recurrence and resistance to cancer therapy is well appreciated. However, therapeutic strategies to target CSCs for cancer therapy has still not progressed sufficiently, particularly in terms of exploring the mechanism of dynamic interconversion between

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CSCs and non-CSCs. Similar to other cells, CSCs also release exosomes loaded with microRNAs (miRs) as part of their paracrine activity. Our review focusses on the exosomal payload of miRs released by cancer cells and their role in the diagnosis as well as prognosis of lung cancer patients.

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INTRODUCTION

Stem cells related to cancer [cancer stem cells (CSCs)] were first reported in 1994 during a study on human acute myeloid leukaemia (AML)^[1]. The authors of the study identified a rare population of AML-initiating cells isolated from AML patients post-engraftment in severe immunocompromised SCID mice. These cells expressed CD34+/CD38- surface markers and were less mature than the colony-forming cells besides possessing a higher proliferative capacity^[1]. Subsequently, in 2003, human CSCs were identified in solid tumours in various organs, including the breast and brain^[2-4]. Hence, a small CSC population (approximately 100 cells) developed into a tumour in an immunodeficient mice model^[2]. A consensus regarding the criteria for classifying CSCs has not been established as yet. However, they are defined as a sub-population in a given tumour with the ability to self-renew and produce cells that can differentiate^[4]. There is growing evidence that CSCs are highly resistant to different types of stresses, including anti-cancer therapy^[5], and hence, they are generally associated with an increased risk of cancer relapse, metastasis and an overall low survival rate^[5]. CSCs have also been linked with tumours identified in various cell lines but not so frequently in all human tissues (*e.g.*, in human lung cancer).

EPIDEMIOLOGY OF LUNG CANCER AND TREATMENT PERSPECTIVES

According to recently published reports, in 2018, there were 121680 new lung cancer cases for men and 112680 for women in the United States, totaling 234030 cases^[6], which is equivalent to an average of 641 lung cancer cases diagnosed per day. These epidemiologic data rank lung carcinoma as the 2nd most prevalent in men behind prostate cancer and for women after breast cancer^[6]. On a positive note, Siegel *et al*^[7] stated that there was an overall 27% decline in cancer-related death cases between 1991 and 2016, translating into more than 2 million fewer deaths than expected if the rate had stayed at the range of the standard values^[7-9]. Conventional therapeutic strategies including surgery, radiotherapy, and chemotherapy are used for lung cancer treatment either singly or in combination at different cancer pathological stages. However, issues associated with chemotherapy and radiotherapy resistance are well known, and recurrence is still a challenge in advanced lung cancer patients. This inability to be 100% curative has been attributed to the sub-populations of stem cells that are capable of self-renewal, undergoing differentiation and producing multi-lineage progenies that may be tumourigenic or non-tumourigenic. This sub-population of cells contributes to the establishment and maintenance of tumours. Although the underlying molecular mechanisms behind these CSCs' properties are less well-defined, the intrinsic resistance of CSCs to therapy is now generally ascribed to a lack of the capacity to induce the apoptotic signalling, increasing telomere length as well as interfering with the cell membrane's ability to act as a transporter, favoring cell migration, and metastasis^[10-12]. Owing to the multifactorial mechanistic nature of resistance, the current treatment methods are inadequate for cancer treatment, and hence, warrant in-depth molecular studies in the future to improve the contemporary therapeutic regimens^[13].

TARGET THERAPY FOR LUNG CSCS

Given their significant role in poor prognosis, relapse and drug resistance in cancer patients^[14,15], the current treatment modalities are being focussed to target CSCs. Some of these emerging treatment modalities include immunotherapy directed against the CSCs' specific surface antigens, interference with signalling pathways (*i.e.*, Notch signalling) and epigenetic approaches^[16,17]. For example, a recent pre-clinical study targeted the self-renewal regulator BMI1 to attenuate CSCs' self-renewal and tumour-initiating potential in oral cavity squamous cell carcinoma^[18]. Based on the promising results during pre-clinical studies, these therapeutic strategies are now entering into the clinical phase of assessment. Another interesting area of research targeting CSCs involves analyzing the underlying mechanisms regulating the dynamic interconversion between CSCs and non-CSCs (Figure 1). An understanding of the molecular mechanism underlying such a bidirectional interconversion of cells would have significant implications on the future development of therapeutic strategies^[19,20].

Besides the CSCs paradigm, the issue of cancer dissemination has been analyzed in-depth with the identification and isolation of microRNAs (miRs). miRs are short (20-24 nucleotides), non-coding RNAs, which are now suggested to be the most promising molecular markers for the diagnosis and prognosis of tumours^[21]. Their regulatory role in various cellular processes is now well-established, although some of the known miRs are dispensable for the normal functioning of cells but have instrumental participation in the initiation as well as the progression of diseases. The main function of miRs is to play an epigenetic role in regulating gene expression at the post-transcription stage^[22]. In the context of their role in cancers, they are grouped as oncomirs that either function as oncogenes or as tumour suppressors. Given the pivotal role of miRs in the physiological functioning of a cell, their dysregulation has been associated with various pathologies, including the initiation and progression of cancer^[23]. Their prognostic value as qualitative and quantitative biomarkers in plasma, either in free form or encapsulated in the microvesicles, has been reported in various cancers^[24]. There have been attempts to classify human cancers based on miR expression profiles^[25].

Exosomes are extracellular vesicles with a small size of approximately 30 to 100 nm. They are formed by the fusion of intracellular components surrounded by the plasma membrane and released from cells^[26]. It is now becoming well-established that exosomes, besides other components, also transfer their nucleic acid payload including miRs, from the cell of their origin to the recipient cells. The importance of exosomes is mainly due to their capacity to transport miRs into the body, and this process forms an important focus for providing a deep understanding of the possible genetic implications between cancer and non-cancer cells^[27]. The miRs thus transferred significantly affect the gene expression and cellular signalling pathways in the recipient cells, including maintenance of a dynamic equilibrium between CSCs and non-CSCs by delivering their miR-payload^[28,29].

Mature miRs that are about 70 nucleotides long are derived from pre-miRs composed of 100 nucleotides and then transcribed^[30]. Exosomes can transport small intracellular components, such as proteins and lipids, which are included by an endocytosis process from pre- to late-mature exosomes^[31]. Hence, exosomes are currently the smallest cellular components carrying miRs from the cell to the human organs. They are detected in many fluids, such as urine^[32,33], blood and saliva, and this peculiarity makes them a unique mediator against tumour development and progression. Each miR has various targets, although different miRs may have a single target^[34], and this characteristic highlights their significant involvement in many genetic and cellular processes, such as in particular the preservation of cellular differentiation. The most interesting characteristic is that many human genes depend on miRs; this aspect reflects the roles of these small molecules in the genome^[35,36].

In recent studies, it has been found that operable non-small cell lung cancer (NSCLC) patients showed a significant association between recurrence and survival^[37-40]. In adenocarcinoma patients, a miR score has been defined for distinguishing between patients in stage I developing recurrence within two years from surgery, and those patients that were disease-free after three years^[41,42]. We believe that determining the roles of exosomes and their miR-payload in the prevention and cure of cancer is the future of personalized cancer medicine. However, targeting exosomes and monitoring miRs in biological fluids will be the pillar for the setting of new approaches in terms of follow-up for cancer patients, with the focus being to determine more details in the history of each person affected from this disease^[43-45].

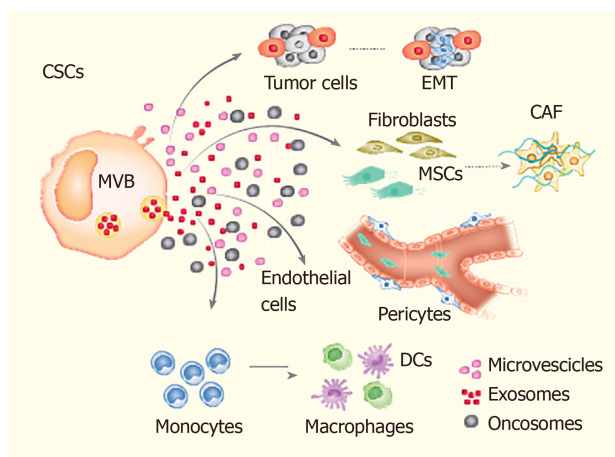


Figure 1 The mechanism of the dynamic interconversion between cancer stem cells and non-cancer stem cells. The cancer stem cells interact with various cell populations of the tumour microenvironment, *i.e.*, tumour epithelial cells, mesenchymal stem cells, endothelial cells, epithelial mesenchymal transitioning cells, fibroblasts, immune cells (monocytes, macrophages *etc.*). This interaction can cause cancer cell stemness phenotype, promotion of tumour cell invasion, angiogenesis and metastasis. EMT: Epithelial to mesenchymal transition; CAF: Cancer-associated fibroblasts; MSCs: Mesenchymal stem cells; CSCs: Cancer stem cells; DCs: Dendritic cells.

CANCER CELL GROWTH, METASTASIS AND ROLE OF EXOSOMAL MIRS

The amount of miRs in exosomes is influenced by the intracellular components as well as by the physiological context^[46-50]. In particular, exosomes can maintain the stability of miRs by preventing their degradation^[48]. The consequence of this exosome-driven role is to drive them in cancer progression as miR-142-3p, miR-150, and miR-451, all of which have been found in exosomes in gastric cancer cells^[46]. Similarly, microsomal miR-21, let-7f, miR-20b, and miR-30e-3p are differentially expressed in patients with solid tumours as compared with healthy people^[46,49]. The role of exosomal miRs is undoubted in terms of cancer growth, development and recurrence. It is apparent that miRs also important players in angiogenesis and metastasis as they can influence host immunity-inducing chemoresistance and tumour microenvironment (TME) re-adaptation^[51].

We know that the metastasis of cancer cells is an intricate process that implicates the colonization of cancer cells from their primary site to a secondary location^[52]. It encompasses a cascade of molecular events that facilitates cell migration, invasion, angiogenesis and epithelial to mesenchymal transition (EMT)^[53]. From amongst the oncomirs, specific miRs are also grouped as “metastamirs” due to their association with the molecular processes and signalling pathways that underlie cancer cell metastasis^[54]. For example, Coebergh *et al*^[55] have recently identified signature miRs, including let-71 and miR-10, which can serve as biomarkers to predict colon cancer patients who are at a risk of metastatic cancer spread. Both miRs’ signatures were successfully used for prediction of hepatic recurrence of cancer in stage-I and -II patients. Similarly, miR-126 suppresses EMT to influence lung cancer cell metastasis^[56]. Molecular studies have revealed the inhibition of the PI3K/Akt Snail pathway with the involvement of miR-126 that could be a potential therapeutic target for lung cancer treatment. Similar observations have also been reported with a possible role of miR-30a *via* targeted regulation of Snail^[57]. Further, the role of Wnt/ β -catenin in EMT has been reported in human colorectal carcinoma metastasis that involved *GNA13* and *PTP4A* genes’ regulation *via* β -catenin signalling and which are targeted by the miR-126 pathway *via* ERK/GSK3 β / β -catenin and Akt/GSK3 β / β -catenin signalling pathways^[58]. The role of β -catenin in EMT has also been reported in a recently published study that involved miR-1246 as a regulator of EMT in A549 cells by inhibiting E-cadherin expression *via* regulation of the Wnt/ β -catenin pathway through GSK3b/ β -catenin targeting^[59]. These data provide vivid evidence for the significant participation of miRs in supporting the metastatic spread of cancers from their primary origin.

There has been a recent interest in miR dissemination through exosomes. In this regard, an important role is played by the cancer-associated fibroblasts into the TME, a process that seems to release exosomes, inducing tumour development or control depending on the presence of some nutrients^[60]. Besides EMT, angiogenesis is

important for tumour maintenance and recurrence. In this context, exosomes released by cancer contribute to increased angiogenesis and tumour growth through the transforming growth factor β 1-dependent pathway, which induces the fibroblast evolution process^[61,62].

In lung cancer, exosomal miR-23a from hypoxic lung cancer cells and hypoxamir-210 from exosomes derived from such cells can improve permeability of the vessel membranes and increase vascularization through the STAT3 mechanism, which can transform normal bronchial cells into malignant ones^[63]. One of the mechanisms that may induce tumour progression involves tumour-derived exosomal interactions with TME. For example, it has been shown that tumour-derived exosomes in lung cancer may induce bone marrow-derived mesenchymal stem cells to change themselves into a phenotype stimulating inflammation^[64]. Hence, the immune system inside TME may be affected by the tumour-derived exosomes with the final result being tumour progression, most likely due to the reprogramming of the immune cells influenced by tumour exosomes^[64-66]. Akin to other cells, the exchange of exosomal miRs from cancer cells to endothelial cells (ECs) significantly influences their angiogenic activity. Tumour cell-released miR-221-3p facilitates lymphangiogenesis in cervical squamous cell carcinoma by its transfer to lymphatic ECs^[67]. Similarly, cancer cell-derived exosomes transfer miR-25-3p to the ECs and regulate VEGF expression by targeting KLF2 and KLF4, thus promoting angiogenesis^[68].

EXOSOMAL MIRS AS BIOMARKERS AND THEIR ROLE IN DRIVING RECURRENCE

As discussed before that the exosomes carrying miRs drive angiogenesis and cancer progression^[69]. For example, it has been shown that miR-103 enhanced angiogenesis and induces tumour metastasis in hepatocarcinoma patients. This process involves several endothelial target proteins, such as VE-cadherin, p120-catenin and zonula occludens 1 in ECs^[70]. In other blood diseases, such as leukaemia, exosomal miR210 secreted by hypoxic leukaemia cells have an important impact on angiogenesis through the receptor tyrosine kinase ligand Ephrin-A3 of ECs^[71]. In contrast, exosomes may include miRs that can harm leukaemia cells, influencing motility and their capacity to adhere. This process is induced by the loss of C-X-C motif chemokine ligand 12 and vascular cell adhesion molecule-1 proteins in ECs^[72].

Several exosomal miRs are essential in the process of recurrence. In particular, in metastatic breast cancer, exosomal miR-210 is involved in EC transport as well as improving angiogenesis^[73]; in nasopharyngeal carcinoma (NPC) cells, miR-23a exosome enhances tumour growth and recurrence^[74], although exosomal miR-9 suppresses NPC cell migration and the consequent vascular formation by targeting midkine and modulating the phosphoinositide-dependent protein kinase/protein kinase B (Akt)-signalling pathway^[75].

Due to their already demonstrated crucial participation in metastatic processes and their presence into human fluids, exosomal miRs are the future of personalized medicine as biological biomarkers^[76]. Exosomal miRs are already in practice as reliable biomarkers for the diagnosis of lung cancer patients^[77-79]. Cazzoli *et al*^[77] performed a thorough exosomal miR-analysis of 30 plasma samples (including $n = 10$ each from lung-adenocarcinoma, lung-granuloma and healthy-smoker subjects) and all the donors were matched for age and sex. The expression level of four miRs distinguished between tumour and healthy-smoker subjects^[77]. These findings were subsequently used on a larger group of patients with 96% sensitivity and nearly 70% specificity. Several upregulated miRs-derived exosomes (such as miR-21 and miR-155) have been found in patients who developed lung cancer recurrence^[78]. In particular, Li *et al*^[63] used a qPCR-based array to analyze plasma from 10 patients affected by lung adenocarcinoma, and he found an increased level of 3 exosomal miRs (miR-23b-3p, miR-10b-5p, and miR-21-5p) which seemed to be correlated with decreased survival^[63]. The expression profile of specific exosomal miRs in lung adenocarcinoma patients vindicated the previously published results that circulating exosomal miRNAs were a useful and possible marker for further diagnostic and therapeutic purposes in lung cancer^[79]. Dejima *et al*^[80] profiled plasma exosomal miRs derived from lung cancer patients to demonstrate that miR-21 and miR-4257 were significantly upregulated as compared with those patients without recurrence and healthy individuals as controls. The microarray data also showed that exosomal miR-21 and miR-4257 exosomes were significantly associated with tumour growth and metastatic invasion in lung cancer. These data were also supported by the low percentage of disease-free survival in patients with high expression of both exosomes levels^[80,81].

Besides the recent reports regarding the pivotal role of exosomal miRs in driving

recurrence and invasion of cancer in patients, their use as biomarkers seems to represent an effective method for diagnostic and prognostic purposes. That is justified by the fact that there are currently no markers that can satisfactorily diagnose presence of tumour in the early stage as well as predict long-term survival with respect to many solid cancers^[82].

Although quantification of a panel of miRs in the plasma or serum of patients at high-risk to develop lung cancer has been proposed, the difficulty in discriminating between miRs for normal and cancerous tissue remains an obstacle in the development of an effective screening method^[83]. Nigita *et al*^[84] analyzed data from 87 NSCLC patients, attempting for the first time to identify and differentiate miRNAs for normal and tumour tissues; in particular, miR-441-5p was the most consistently detected among the NSCLC exosomes.

One of the mechanisms by which exosomal miRs affect the functioning of CSCs is *via* regulation of the interaction between CSCs and their microenvironment^[85]. The exosomes serve as carriers of the genetic information (*i.e.*, miRs) required for regulation of the signalling involved in the transformation of cancer cells into CSCs to achieve a dynamic equilibrium between the two cell types.

EXOSOMAL MIR PAYLOAD AND TME

Reflecting their role in metastatic processes, it is apparent that exosomal miRs have an important impact on TME^[86]. It has been demonstrated that exosomes driven by specific miRs, such as miR-223 derived from macrophages, may induce drug resistance in the hypoxic TME^[87].

The interactions between cancer cells, exosomal miRs, and the TME rely on a complex network that has yet to be satisfactorily clarified. In particular, exosomes can mediate immune regulation, and several studies are attempting to understand the direct effects of exosomes in T-cell activation^[88]. However, there is currently a lack of understanding regarding the identification of the connections between the immune system, miRs, exosomes, and CSCs. Recently, a correlation between tumour-infiltrating lymphocytes (TILs) and CSCs in NSCLC patients has been demonstrated^[89]. This is important to highlight the existence of interactions and relations of these cellular components to further connect exosomes carrying miRs, CSCs and TME. Further clarification of the associations between TILs and CSCs will be helpful for the development of targeted therapies that may focus on miR exosomes, CSCs, and TME cells.

The complexity of TME leads to difficulty in understanding the mechanisms involved. TME is an amalgam of both cellular and non-cellular components that encompasses the surrounding microvascular structures, immune cells, fibroblasts, ECs, cancer cells, signalling molecules and the extracellular matrix (ECM) (Figure 2). Put together, these components offer a growth factor and cytokine-rich TME that is conducive for phenotypic plasticity, immune surveillance, survival, angiogenesis and cancer cell metastasis^[90,91]. More importantly, TME contributes significantly to the spatiotemporal dynamics of pattern formation and is one of the primary factors that substantially influence tumour heterogeneity^[92]. The link between TME and CSCs generation is represented by the EMT^[93].

The discovery of the TME implies the possibility of a novel treatment strategy that goes beyond the paradigm of cancer genetics that restricts its focus only on cancer cells^[94]. The presence of CSCs in the TME is crucial for 'tumour nutrition' due to their capacity to reproduce themselves, inducing tumour growth in NOD-SCID mice and facilitating the spread and chemoresistance of the tumour. There is mounting evidence that conservative therapeutic strategies (*i.e.*, radiotherapy and chemotherapy) largely fail to eliminate CSCs, which are now associated with minimal residual disease and cancer relapse^[95,96].

The physiological functioning of cells is affected by a multitude of physical factors that alter both genetic and epigenetic states. These molecular changes influence the intracellular regulatory circuitry that enables the body to achieve as well as sustain an appropriate response to the changing environment^[97-99]. In this regard, ECM is an elastic barrier able to change the mechanical properties of its proteins through genes' profile mutations. Studies of the TME have led to immunotherapy and other new-generation immune therapies, such as CAR-T cells^[100-102] and anti-cancer vaccines^[103-105]. The mechanism underlying the role of TME-associated transformation of normal cells into tumour cells and then converting to malignancy upon cancer initiation is less well defined^[94]. However, this mechanism is associated with the mechanical/scaffold (elasticity) effect of ECM^[106]. Li *et al*^[94] suggested that the elasticity is an important aspect in ECM development, playing a key-role in miR exosome expression,

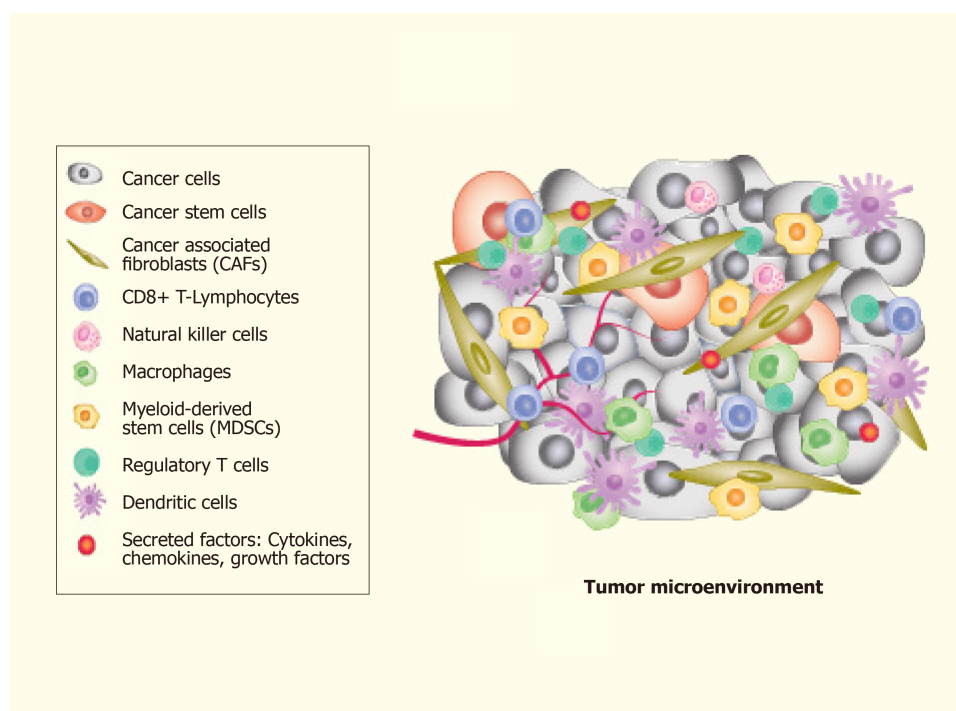


Figure 2 The components of the tumour-microenvironment. The cellular components of the tumour-microenvironment include different types of cells including such as epithelial tumour cells, cancer stem cells, and immune cells. Every type of cellular component contributes to maintain the tumour alive and to develop neo-vessels for promoting the tumour cells dissemination.

regulating tumour gene expression and tumour growth. Further understanding of the role of tumour-TME interaction will significantly help in understanding the acquired resistance of cancer cells towards contemporary cancer therapies including surgery, radiotherapy, chemotherapy and anti-angiogenic therapy^[107]. This understanding will open up a new avenue to target the CSC niche within the TME. In this context, in particular, the exosomes derived from the tumour (TEX) seem to have a strong influence on the TME for their capacity to expand themselves in other tissues and contributing to cancer dissemination to the organs^[108]. Additionally, besides TME, Javeed *et al*^[109] hypothesized the presence of a tumour “macroenvironment” (TMAE) that results from the pathological interaction between tumour cells with other organs and systems of the body to mediate immunosuppression and promote genetic and metabolic reprogramming of the cells through exosomal-miR payloads^[109]. Further understanding of the TMAE would be helpful for two reasons: first, it would help in identifying those patients who would benefit from systemic therapy and second, it would help in the future development of novel systemic therapies^[110].

One of the most interesting aspects in this context is that TEX is associated with some antigens, such as programmed death-ligand 1 (PD-L1), which induces the pre-metastatic pathway for tumour dissemination^[111]. PD-L1 expression has been reported to be enhanced by exosomes from melanoma, and this justifies the roles of exosomes in tumour growth, metastasis and immune modulation^[112,113]. There is interest in establishing an “exosome screening” approach due to their presence in many fluids of the human body, and this aspect is highly important in terms of providing potential biomarkers as well as drug carriers for targeted drug delivery^[114-116].

THE POTENTIAL ROLE OF MIRS IN TUMOUR AGGRESSIVENESS

“Tumour aggressiveness” is typically used to define a highly incurable end-stage cancer that is also able to resist the standard treatments^[117-119]. Dysregulated miRs can be oncogenes (oncomirs) if upregulated, or tumour suppressors (anti-oncomirs) if downregulated, depending on their target transcripts^[120,121]. Tumour aggressiveness is generally associated with altered miRs profile that has already been reported in various solid tumours^[121-125]. Specifically, miRs have been implicated in a substantial number of intracellular mechanisms that include tumour growth, proliferation, recurrence, tumour metabolic alterations and chemo- and radio-resistance. However,

they are also influenced by many extracellular factors, such as hypoxia, which seems to contribute to poor clinical outcomes by increasing the recurrence risk^[126-128].

The mechanism of oxygen sensing at the cellular level has remained an area of intense investigation, as evidenced by the two Nobel Prizes won by Otto Warburg in 1931 and Corneille Heymans in 1938 for their findings on the role of enzymes and the nervous system in respiratory cellular mechanisms^[126]. Moreover, the genetic component of adaptation to oxygen flux remained oblivious for the major part of this century. More recent studies have successfully addressed this issue to elucidate the molecular biology of hypoxia-inducible factor (HIF) signalling as part of the body's hypoxic response in health and disease^[127,128]. HIF-1 signalling and HIF-1-dependent miRs, hypoxamirs, are being extensively studied for their role in cell survival and angiogenesis in the context of cancer cell biology^[129-132] as well as regenerative medicine^[133]. The data thus generated has been exploited to promote stem cell survival and angiogenesis either as part of physical or genetic manipulation of cells for hypoxamir expression^[134-138]. In this context, the homeostasis of reactive oxygen species (ROS) is the key for maintaining normal biological processes^[139]. Higher-level oxidative stress produces irreversible damage to intracellular and cellular components, thus altering the stability of the genome with the induction of malignant cell transformation^[140]. For example, altered production of ROS is also associated with EMT, tumorigenesis and tumour progression^[141]. In particular, the mechanism of ROS on CSC mechanism regulation is not yet fully clarified.

The precise mechanism by which ROS regulates CSCs and EMT characteristics with the HIF-mediated pathways is unclear^[140-142]. It has been documented that normal stem cells, as well as CSCs, have a low level of ROS, potentially due to their strong defense system against DNA damage^[140-143]. Low-level ROS in CSC-like cells is associated with higher free radical scavenger production. The inhibition of ROS scavengers by drug treatment in mouse breast Thy-1 + CD24 + Lin- CSC-enriched cells markedly decreased their clonogenicity with increased radio-sensitization^[144]. Hence, low levels of ROS and enhanced ROS defense may contribute to tumour radio-resistance as compared to non-tumorigenic counterparts^[145]. These findings strongly suggest that ROS may play an important role in the pathogenesis of CSCs.

Surprisingly, there is a link between ROS and miRs. It has been demonstrated that H₂O₂ treatment can dysregulate the expression of certain miRs in vascular smooth muscle cells and macrophages. Moreover, it has also been demonstrated that miR-30e contributes to regulating oxidative stress and ROS levels through SNAIL mRNA in human umbilical endothelial vein cells^[146]. Given the significant regulatory role of miRs in metabolic processes in the cells, many of the oncomirs, such as miR-21, are directly involved in the formation of ROS and hence, promote tumorigenesis^[147]. Both ROS and miRs control each other's expression in cancer cells to maintain a balance that is supportive of cancer cells in terms of their ability to produce the hallmarks of cancer^[148,149]. More investigations are mandatory to clarify the possible importance of ROS in CSC regulation.

CLINICAL IMPLICATIONS

Although recent studies have identified the presence of CSCs in ADENO and SQUAMO cell carcinomas of the human lung, with this aspect being important in terms of understanding that CSCs are present in all NSCLC tumours^[150-152], even in neuroendocrine tumours of the lung^[153], the possibility to target CSCs is still debated. In particular, the scientific community is focusing attention on the characterization of miR exosomes for their intrinsic characteristics as complex paracrine factors^[153]. In fact, it has been found that stem cell-derived exosomal miRs can be used to modulate the therapeutic response to stroke and may increase their therapeutic potential^[77]. This aspect may be important in future if we consider the possibility of targeting CSC-derived exosomal miRs, in consideration of CSCs' characteristics related to chemo- and radioresistance – this approach would have a key role in new cancer treatments.

Lung cancer cell-derived exosomal miRs are at the centre of interest in the present-day research for future roles as predictors of recurrence as well as biomarkers in the early stage or for their prognostic role in advanced-treated patients^[13,154-157]. The prime objective is to explore the possibility of their use as biomarkers for early diagnosis of lung cancer combined with target therapy^[158,159]. In one of the studies, immunostaining of exosomes from lung cancer tissue and chronic lung disease showed that 80% of these specimen-derived exosomes were positive for EGFR, although 2% of the inflammatory tissue was positive. This point suggested a possible role of EGFR exosomes as potential biomarkers in lung cancer^[61,160,161]. A similar result has also been identified in ALK-EML4 translocated exosomes, which are markers for first-

generation treatment ALK-TKIs^[162,163].

On the same note, exosomes from the plasma of NSCLC patients ($n = 276$) indicated that exosomes may have a prognostic role as biomarkers in NSCLC^[156]. Similar results were found in plasma exosomal miRs analyzed from lung adenocarcinoma patients ($n = 84$) versus healthy controls. These results showed increased levels of exosomal miR-10b-5p, miR-23b-3p, and miR-21-5p were significantly associated with poor prognosis, thus suggesting their significance as biomarkers of NSCLC prognosis^[157]. The use of exosomal miRs as prognostic biomarkers is the basis for new-generation target therapy against NSCLC, particularly in lung cancer for highly important proteins such as EGFR, KRAS and RAB family.

The most relevant aspect related to targetable exosomes is connected to the precision treatment of NSCLC^[164]. In this regard, exosomes can be produced as new cellular molecules of delivery for medical treatment as well as against tumours to reduce the negative collateral effects in the human body^[165,166]. Lai *et al*^[167] have established a method for loading exosomes with a drug or genetically manipulating cells with genes of interest to alter the exosomal payload derived from these cells. Mendt *et al*^[168] have reported a standard operating procedure to engineer exosomes that could target KRAS (iExosomes), with a particularly good response in terms of improved survival. The possibility to target genes such as KRAS is highly innovative because it is responsible for the highest mortality rate in NSCLC patients.

The diversity of exosomal-payload and their functions relevant to NSCLC may provide future pioneering target treatments. Yang *et al*^[169] determined that induced expression of miR-let-7 in exosomes for NSCLC treatment was specific and effective in tumour suppression^[169,170]. Similarly, exosomes isolated from H460 cells and that had been transduced for the restoration of LKB1 (liver kinase B1) had an increased ability to restore lung cancer cell migration as compared to exosomes isolated from H460 cells lacking in LKB1 activity^[171]. While elucidating the process, it was observed that H460 cells with restored LKB1 supported the emigrational activity of lung cancer cells by the suppression of exosomal secretion of migration-suppressing miRs, including miR-125a, miR-126a, and let7b. These data highlight the significance of LKB1 as a new target for future cancer therapy. In an interesting new development, antibody therapy with anti-CD9 or anti-CD63 to target tumour-derived exosomes effectively inhibited the progression of breast cancer in mice, suggesting a successful further treatment in lung cancer^[172].

CONCLUSION

Characterization of CSC-derived exosomes in terms of their payload and the use of exosomes for CSC targeting have emerged as potential strategies for cancer theranostics. For example, the safety and feasibility of exosome targeting were first assessed in phase I clinical trial for metastatic melanoma^[173]. The patients were treated with autologous dendritic cell-derived exosomes (DEX) loaded with the melanoma antigen gene (MAGE) by intradermal and subcutaneous^[173,174]. Although no significant outcome was reported, the data vividly demonstrated that exosome-based treatment may represent a new approach for curing cancer patients. The results from phase I trial evidenced that the immune system of treated patients was active and that exosome-treated patients showed limited disease progression^[174]. Based on these encouraging data, DEX has progressed to Phase II trials as maintenance immunotherapy after first-line chemotherapy in NSCLC patients^[175]. Besides the value and importance of exosomes as mediators against anticancer effects, it is necessary to study their clinical effects in the human body to guarantee a better standardization of the methods of processing and ensure optimal and reproducible anti-tumour immune responses after exosome-based therapy in the clinical perspective^[176,177].

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Stem cell-based approaches: Possible route to hearing restoration?

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Abstract

Disabling hearing loss is the most common sensorineural disability worldwide. It affects around 466 million people and its incidence is expected to rise to around 900 million people by 2050, according to World Health Organization estimates. Most cases of hearing impairment are due to the degeneration of hair cells (HCs) in the cochlea, mechano-receptors that transduce incoming sound information into electrical signals that are sent to the brain. Damage to these cells is mainly caused by exposure to aminoglycoside antibiotics and to some anti-cancer drugs such as cisplatin, loud sounds, age, infections and genetic mutations. Hearing deficits may also result from damage to the spiral ganglion neurons that innervate cochlear HCs. Differently from what is observed in avian and non-mammalian species, there is no regeneration of missing sensory cell types in the adult mammalian cochlea, what makes hearing loss an irreversible process. This review summarizes the research that has been conducted with the aim of developing cell-based strategies that lead to sensory cell replacement in the adult cochlea and, ultimately, to hearing restoration. Two main lines of research are discussed, one directed toward the transplantation of exogenous replacement cells into the damaged tissue, and another that aims at reactivating the regenerative potential of putative progenitor cells in the adult inner ear. Results from some of the studies that have been conducted are presented and the advantages and drawbacks of the various approaches discussed.

Key words: Hearing loss; Cochlear hair cells; Spiral ganglion neurons; Cell regeneration; Adult stem cells; Cell transplantation

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Core tip: This review summarizes the various approaches that are being explored to establish cell-based therapies that tackle the irreversible loss of sensory cells in the adult cochlea. Advantages and disadvantages of the various approaches are discussed, based on published results, and some considerations are made on future perspectives, taking into account the new developments in the field.



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INTRODUCTION

Hearing loss is the most frequent sensorineural impairment in man; it affects over 6% of the world's population and its incidence is expected to rise to around 900 million people 10% of the population by 2050 [World Health Organization (WHO) estimates, <https://www.who.int/pbd/deafness/estimates/en/>]; thus this disability poses very serious social and economic implications. Most cases of hearing impairment are due to degeneration of sensory hair cells (HCs), the mechano-receptors in the inner ear; damage to these cells may be caused by clinically administered drugs, such as aminoglycoside antibiotics and some chemotherapeutics (e.g., cisplatin), exposure to high levels of sound, aging, infections, some occupational hazards, genetic mutations. A smaller percentage of the cases are due to damage to the spiral ganglion neurons (SGNs) that innervate the HCs in the cochlea and act as the first relay in the transmission of the incoming sound information to the brain. Differently from the case in avian and other non-mammalian species, regeneration of missing cell types does not occur in the mammalian cochlea, and the ensuing hearing loss is thus permanent. Various approaches to cell regeneration are being explored, namely gene therapy, administration of survival factors and other biologically active molecules, and cell-based replacement strategies^[1-5]. With regard to the latter studies, there are two main lines of research, one that is based on the introduction into the damaged cochlea of exogenous replacement cells, and another that aims at reactivating the regenerative potential of putative progenitor cells in the adult inner ear.

GENERATION OF OTIC CELL TYPES FROM EXOGENOUS CELL SOURCES

When considering the implantation of exogenous cells into the cochlea, a wide range of factors must be taken into account that have an important effect on the fate of the implanted cells and ultimately determine treatment outcome. Considerations such as the type of exogenous cells, their differentiation status and potential to differentiate towards other cell types, the selected route of implantation and the host micro-environment all play key roles in the survival and integration of the implanted cell population within the host tissue (Figure 1).

Tissue-specific progenitor cells

There are numerous studies reporting on the potential of different exogenous cell types to replace sensory cells in the damaged inner ear. Some of the work has focused on cells that, although coming from other tissues, may still share some relevant characteristics with otic cell types. Wei *et al*^[6] demonstrated the capacity of ciliated ependymal cells, obtained from the forebrain germinal zone of adult mice, to get incorporated into streptomycin-treated cochlear explants, express markers typical of HCs and establish functional synapses with primary auditory neurons. In addition, the authors presented data indicating that neural stem cells (NSCs) isolated from the subventricular zone could be differentiated *in vitro* to neurons that established functional contacts with denervated HCs and with adult SGNs in corresponding co-cultures. *In vivo* work carried out by Hu *et al*^[7] demonstrated significant migration of adult mouse NSCs transplanted into adult guinea pig cochleae to relevant locations such as the organ of Corti (OC), the spiral ganglion and the auditory nerve tract. However, NSC survival rates were very low; neomycin-induced damage to the cochlea and also *Neurogenin2*-transduction of the NSCs prior to transplantation improved differentiation of the transplanted NSCs towards a neuronal phenotype and increased NSC survival. Higher rates of cell survival were registered by Regala *et al*^[8] following transplantation of murine adult NSCs into surgically damaged vestibulocochlear nerves in adult rats; importantly, the authors observed migration of murine cells to the brain stem in 50% of the transplanted animals. In another series of experiments, Edin *et al*^[9] obtained SGN-like cells from human neural progenitors (NPs) by applying culture conditions used to maintain primary cultures of guinea pig

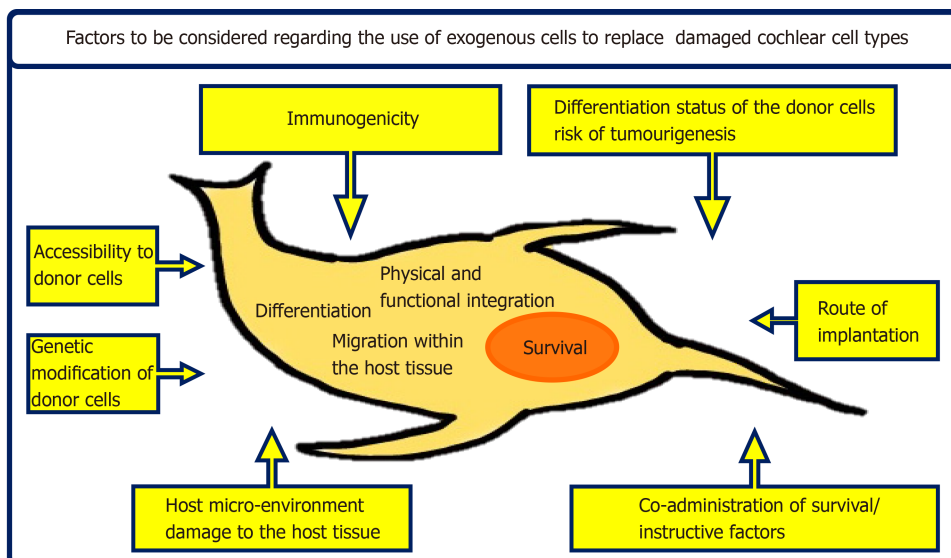


Figure 1 Factors to be considered regarding the use of exogenous cells to replace damaged cochlear cell types. The ultimate fate of implanted exogenous cells, regarding their survival, differentiation towards desired cell phenotypes, their migration to relevant locations in the cochlea and central auditory targets, and their physical and functional integration within the host tissue, strongly depends on a variety of factors such as the type of donor cell, its differentiation status and differentiation potential, the route of implantation and the host micro-environment, among others.

and human SGNs; the cells developed morphological features and expressed an array of markers typical of SGNs.

Although NSCs from the subventricular zone could be used in autografts, thus overriding the need for immunosuppression, their isolation poses a series of technical issues. Therefore, other alternatives have been studied, such as the use of precursor cells that are resident in the olfactory epithelium^[10,11], a readily accessible tissue. These precursors exhibit stem cell-like properties and express markers typical of otic HCs (MYOSIN VIIA, CALRETININ, ESPIN, PRESTIN) when co-cultured with cells from adult cochlea or when exposed to cochlea-conditioned medium^[10]. Promising results were obtained by Xu *et al*^[12], when implanting olfactory epithelium NSCs into the scala tympani of a rat model of noise-induced hearing loss; the implanted NSCs survived and migrated towards host spiral ganglion neurons, although they did not reach the OC. Yet the authors registered improved ABR results in NSC-treated deafened animals, compared to those from non-implanted deafened controls.

Notwithstanding, inner ear progenitors have been isolated from embryonic^[13,14] and adult human tissues^[15] that would be highly suitable donor cells; however, this is not a likely option, given their very low numbers. Interestingly, Stefan Heller and his group have shown that it is possible to isolate cochlear stem cells from post-mortem tissues without loss of their self-renewing and differentiation potential during the first 10 days following death^[16].

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) constitute another candidate donor cell source^[17-21]. These are readily accessible cells with potential to differentiate into multiple lineages, which makes them highly desirable candidates for autologous cell-based therapies; importantly, they exert immunosuppressive properties^[22] and it has been recently shown that transtympanic MSC administration into immunocompetent rat hosts does not elicit an inflammatory response^[23]. Kondo *et al*^[17] demonstrated the potential of murine MSCs (mMSCs) to acquire features of post-mitotic neurons following exposure to sonic hedgehog and retinoic acid (RA); the cells expressed a whole range of glutamatergic sensory neuron markers (SOX10, GATA3, GLUR4, VGLUT1, CALRETININ). An additional soluble protein present in hindbrain/somite/otocyst-conditioned medium and also in embryonic day 18 (E18) OC was required to induce the expression of additional sensory neuron markers (*Brn3a*, *Neurogenin1*, *NeuroD*). Further work by this group^[24] demonstrated that Wnt signalling induces the expression of a whole array of sensory neuron markers through the up-regulation of T cell leukemia 3 (*Tlx3*), a transcription factor that promotes differentiation towards glutamatergic phenotypes. Infusion of Wnt1 was shown to increase the survival and engraftment rates of mMSCs implanted into the modiolus of ouabain-treated Mongolian gerbils^[24]. Moreover, the implanted cells migrated throughout all the cochlear turns and reached the spiral ganglion; some of the cells adopted a clearly

neuronal morphology and expressed neuronal markers.

In another series of experiments, Jeon *et al*^[18] demonstrated that mMSCs could be differentiated to HC-like cells by exposing the mMSCs to a culture regime used to differentiate mouse embryonic stem cells (mESCs) into HC-like cells^[25] and then over-expressing *Math1*, a well-known HC master gene^[26]. mMSCs were differentiated to neurosensory progenitors (mMSC-NsPs) that expressed markers of early otic development, such as *Otx2*, *Nestin*, *Sox2*, *Musashi*, the early HC genes *Math1*, *Brn3c* and *GATA3*, and sensory neuronal markers such as *TrkB* and *TrkC*, among others. Transfection of mMSC-NsPs with *Math1* induced expression of the HC genes *MyosinVIIa* and *Espin*, and other supporting cell (SC) and neuronal markers. Differentiation to a HC phenotype was promoted by co-culturing non-transfected mMSC-NsPs with E13 chick otocyst cells and also by injecting these cells into chick otocysts; of note, the injected cells integrated into chick otic epithelia, especially at sites of damage. Interestingly, when similar studies to those carried out by Kondo *et al*^[17] and Jeon *et al*^[18] were conducted on human MSCs (hMSCs), some differences were observed in the response of these human cells to differentiation cues, compared to that of their murine counterparts^[19,20]. Therefore, Durán-Alonso *et al*^[20] applied to hMSC cultures protocols that had been employed to direct human foetal auditory stem cells (hFASCs) from the cochleae of 9-11-week-old fetuses^[27] towards HC and auditory neuron fates^[28]. hMSCs were initially differentiated to neural progenitors (NPs); further treatment of hMSC-NPs resulted in induced expression of combinations of HC or SGN markers, depending on the culture regimes being applied. HC marker expression (*ATOH1*, *MYOSINVIIa*, *BRN3C*, *CALRETININ*) was only observed following treatment of hMSC-NPs that had been generated in suspension cultures, pointing to differences in the NP populations that had been obtained in floating and in adherent cultures. Unlike the results obtained by Jeon *et al*^[18] on mMSCs, co-culture of hMSC-NPs with chick otocyst cells did not promote differentiation towards the HC lineage. Expression of sensory neuron markers (*SOX2*, *GATA3*, *NGN1*, *ISLET1*, *NF200*) could only be induced in hMSC-NP cultures that had undergone RA treatment, thought to render the cultures responsive to subsequent differentiation cues, such as bFGF. Additional experiments demonstrated that co-culture of 3D hMSC-NPs with murine cochlear explants promoted the expression of the neuronal marker NF-200 in these cells. Differently from the protocols applied by Durán-Alonso *et al*^[20], based on the use of defined media containing specific growth factor combinations, Boddy *et al*^[19] exposed hMSC cultures to media conditioned by hFASCs and observed sequential up-regulation of otic progenitor (*PAX8*, *PAX2*, *SOX2*), HC (*ATOH1*, *BRN3C*, *MYOSINVIIa*) and sensory neuron (*NGN1*, *BRN3A*) markers over time. A role was demonstrated for *Wnt* signalling at the early stages of otic induction. Additional work by Bas *et al*^[21] demonstrated the capacity of human nasal MSCs to integrate into gentamicin-treated cochlear explants from post-natal rats, mostly in the spiral ganglion region; MSCs did not integrate into undamaged explants. Higher numbers of cells expressing β III-TUBULIN were observed in cultures of damaged cochleae that had received hMSCs compared to those that had not been cultured with the human cells; over half of these neurons were hMSC-derived, indicating both differentiation of the exogenous cells and a protective effect on remaining SGNs. hMSC-derived neurons were excitable, and projected neurites towards the sensory epithelium, further promoted by *Wnt* signalling activation. In a different set of experiments, Schäck *et al*^[29] explored the possibility to direct the differentiation of hMSCs to a glutamatergic neuron phenotype by conditionally expressing *Ngn1*, as already demonstrated by Reyes *et al*^[30] on mESCs. hMSCs were refractory to adopting the desired fate as, although some glutamatergic neuronal markers were induced over time, their expression was not maintained once conditional expression of *Ngn1* was halted^[29].

ESCs and induced pluripotent stem cells

***In vivo* survival and differentiation of transplanted stem cell types:** Notwithstanding the valuable data obtained on the various types of exogenous cells mentioned above, the main advancements in the field have come from exploiting the great proliferative and multilineage differentiation potential offered by ESCs and induced pluripotent stem cells (iPSCs)^[1,5,31]. Experiments have been carried out to investigate the influence of the host environment on the survival and differentiation of transplanted stem cells. Survival and induction of neuronal marker expression have been demonstrated at various timepoints following implantation into various *in vivo* animal models^[8,32,33]. Additionally, some of these cells were seen to migrate to relevant locations such as the brain stem^[8,32,33]; of note, work by Zhu *et al*^[33] reported teratoma formation in a number of recipient cochleae following transplantation of murine iPSCs (miPSCs). Genetic modification of donor stem cells prior to their implantation in order to favour their *in vivo* survival and/or differentiation has also been carried

out^[30]. An example of this is the work carried out by Reyes *et al*^[30]; transient *Ngn1* expression in mECSs following their implantation into the scala tympani of kanamycin-treated guinea pigs resulted in increased migration and neuronal differentiation rates, compared to those of mESC controls^[30].

In vitro differentiation. Effect of the otic micro-environment on differentiating cell types: Another line of work has pursued the *in vitro* differentiation of ESCs and iPSCs towards inner ear sensory cell types. A major breakthrough came from the observations by Stefan Heller's group^[25] that sequential incubation of mESC-derived embryoid bodies in serum-free medium (SFM) containing combinations of EGF, IGF-1 and bFGF resulted in the emergence of inner ear progenitor cells in the cultures; cells expressed markers that are seen during otic vesicle formation, such as *Nestin*, *Otx2*, *Pax2*, *Bmp7* and *Jagged1*. In order to promote further maturation of these progenitor cells, the cultures were maintained in defined medium following withdrawal of growth factors; early markers *Nestin*, *Pax2* and *Bmp7* were downregulated, while the expression of HC genes such as *Math1*, *Pou4f3*, *Jagged1*, *Myosin VIIa*, *Parvalbumin*, *AchRα9*, *p31^{Kip1}* was induced; *Espin* was also expressed, indicative of stereociliary morphogenesis. Timing of expression and co-expression patterns of the various genes supported the hypothesis that cultures were mimicking *in vivo* inner ear developmental stages. Importantly, the authors demonstrated integration of inner ear progenitors into developing chick otic epithelia. Integration preferentially occurred in areas of the epithelium that had been damaged during surgery; progenitor cells that incorporated within HC-bearing regions up-regulated the HC marker MYOSIN VIIA and some developed F-actin-rich hair bundles that were labelled with an anti-ESPIN antibody, demonstrating an instructive role of the otic environment. The same group later developed a more elaborate step-wise approach to differentiate mESCs and miPSCs^[34], where ectodermal induction was promoted at the expense of endoderm and mesoderm, and the formation of anterior ectoderm was favoured by the addition of IGF1; FGF combinations were then applied as the main otic inductive signals. Removal of the growth factors present in the medium^[25] resulted in *Math1* and *Myosin VIIa* expression; however, the cells did not present the typical HC morphology nor were hair bundle markers such as *Espin* detected. These features were only observed when the otic progenitor cells were grown on a layer of mitotically inactivated E18 chicken utricle stromal cells; the cells in these cultures exhibited stereociliary bundles and responded to mechanical stimulation in similar ways to those of immature HCs. Heller's group then extended their studies to hESCs^[35], applying to their cultures a modification of the treatment regime used for mESCs and miPSCs. The number of cells shown to express a combination of various HC markers was low and the cells resembled nascent HCs that did not further mature by increasing culture times but died instead. Additional studies were carried out^[36] on monolayer cultures of hESCs and hiPSCs, in an attempt to better characterize the conditions required to obtain *bona fide* otic cell types from these cultures; this work identified retinoic acid as a critical factor for bFGF-induced expression of early otic markers in pre-placodal ectoderm cells. Nevertheless, no further differentiation of the cells was attained, indicating that the monolayer culture model lacked some of the factors found in aggregate cultures that promote the differentiation of otic progenitor cells. Supporting these findings, Abboud *et al*^[37] obtained better results when applying an otic induction protocol [modified from (34)] to mESC cultures grown in floating conditions compared to cultures grown as monolayers. A greater proportion of the cells grown under non-adherent conditions expressed otic progenitor (PAX2, SIX1, EYA1, SOX2) and early HC markers (MYOSIN VIIA, POU4F3) following treatment, compared to adherent cultures. Following induction, floating cultures were partially dissociated and grafted into neomycin-damaged murine cochlear explants; a small number of these cells survived and integrated into the host tissue, preferentially in damaged areas of the OC, and expressed MYOSIN VIIA. Interestingly, this was not observed for any of the progenitors that had integrated outside the lesioned area. Notwithstanding, attempts have been made to conduct otic induction experiments on cell monolayers, rather than on three-dimensional cultures that are prone to higher variability^[13,38]. Marcelo Rivolta's group^[13] obtained otic progenitor cells (expressing PAX8, SOX2, FOXG1, PAX2, NESTIN, SIX1 and GATA3) following 10-12-day-culture of hESC monolayers in SFM containing a combination of FGF3 and FGF10 or combinations of EGF, IGF-1 and bFGF factors^[25]. The authors described two different types of colonies, large epithelioid colonies, composed of flat cells of large cytoplasm (otic epithelial progenitors, OEPs) and smaller colonies, formed by cells that presented denser chromatin and cytoplasmic projections (otic neural progenitors, ONPs). HC-like cells that co-expressed various HC marker combinations were obtained from OEPs following culture in SFM containing EGF and RA. A similar protocol was applied by Chen *et al*^[39] to generate OEPs and ONPs from hiPSCs. OEPs were grown

on mitomycin-treated chicken embryonic utricle stromal cells, in SFM containing EGF and RA, to yield rates of over 40% of cells that co-expressed HC markers (BRN3C, MYOSIN VIIA and ATOH1) and demonstrated some other characteristics of HCs such as the presence of mechano-transduction channels and some electrophysiological activity. In *in vitro* co-cultures of OEP-derived HC-like cells and SGNs from neonatal mice, SGNs extended neurites to the induced HC-like cells and formed active synapses. Additionally, OEPs were transplanted into the scala tympani of *Slc26a4*-null mice that present HC defects. At 4 wk post-transplantation some cells had migrated to the scala media and had integrated into the damaged epithelium, expressing MYOSIN VIIA and forming synaptic connections with native SGNs. The same protocol was applied by Azel Zine's group^[38] to obtain HC-like cells from hiPSCs; otic induction was significantly increased when the EGF/RA step was substituted by treatment with a Notch inhibitor, in agreement with accumulated evidence that Notch plays a key role in the differentiation of sensory otic lineages. Thus ATOH1 expression was much higher in cultures exposed to the Notch inhibitor and around 50% of the cells in these cultures expressed MYOSIN VIIA, as opposed to cultures grown in the presence of EGF/RA, where the percentage of cells expressing this HC marker did not reach 5%. Unfortunately, no hair bundle formation was detected on differentiating cells. Very importantly, hiPSC-derived otic progenitors could survive in an *in vivo* ototoxic damage model^[40]. The cells were implanted into the cochlea of adult guinea pigs that had undergone amikacin treatment. Two weeks after implantation, surviving progenitors had engrafted within the damaged cochlear sensory epithelium and expressed MYOSIN VIIA; some expressed SOX2, pointing at their differentiation towards a SC type. Interestingly, those progenitors that had integrated outside the area of the OC did not express MYOSIN VIIA. Similar results were obtained when implanting murine otic progenitors into the same *in vivo* model.

In addition to the protocols discussed above, other HC induction protocols have been described. An example is provided by the work by Ouji and colleagues^[41,42], based on the culture of mESC-derived embryoid bodies in medium conditioned by ST2 stromal cells. This treatment led to the induction of HC marker expression and the formation of stereocilia-like structures in some of the cells; additionally, some cells were shown to integrate into developing chick otocysts. A simpler method was developed by Ohnishi *et al*^[43] in an attempt to eliminate the need for conditioned media, complex growth factor combinations, or the use of xenogeneic cells. They reported expression of MYOSIN VIIA and β III TUBULIN proteins in hiPSC cultures that had been grown in defined medium, using bFGF as sole growth factor; stereocilia-like protrusions were observed in some MYOSIN VIIA-expressing cells. Although simpler than other methods, induction rates were extremely low.

Differently from the methods described above, Domingos Henrique's group^[44] directly programmed mESCs to become HCs by forcing the simultaneous expression of *Gfi1*, *Pou4f3* and *Atoh1* (GPA), coding for three key transcription factors in HC development. Theirs was an extremely fast and efficient induction protocol that in 8-12 days yielded large numbers of cells ($54\% \pm 2\%$) that co-expressed various HC markers. Addition of RA or inhibition of the Notch pathway during GPA overexpression resulted in increased HC induction rates ($84\% \pm 1\%$ and $70\% \pm 2\%$, respectively). Some maturation of the MYOSIN VIIA+ cells were observed from d8 to d12, indicated by a decline in SOX2 expression and clear expression of the hair bundle proteins ESPIN and CADHERIN23 in membrane protrusions that did not reach the degree of organization found in normal HC stereociliary bundles. Nevertheless, FM1-43 incorporation experiments pointed at the presence of potentially functional mechano-transduction channels. Reyes *et al*^[30] also resorted to genetic modification of mESCs in order to guide their differentiation *in vitro* and attained high rates of differentiation of mESCs to glutamatergic neurons through the transient expression of *Ngf1* in the cultures.

ESCs and iPSCs have also been differentiated *in vitro*^[13,45] and *in vivo* towards SGNs. Some of the work has consisted on generating stem cell-derived NPs that have then been implanted in the inner ear to promote their differentiation towards the SGN lineage. An example of this approach is the work carried out by Corrales *et al*^[46], who grafted mESC-NPs into the cochlear nerve trunk of ouabain-treated gerbils. Implanted cells survived and demonstrated β III TUBULIN and PERIPHERIN expression; interestingly, they extended processes towards the denervated HCs in the OC, indicating a role of the host environment as provider of survival, differentiation and guidance cues. Unfortunately, no functional recovery could be demonstrated. Coleman *et al*^[47] implanted mESC-NPs into the scala tympani of chemically deafened guinea pigs, selecting a delivery route that was clinically more relevant than others previously used, such as direct injection into the auditory nerve. Transplanted cells were observed in the scala tympani of transplanted hosts at 4 wk post-transplantation. mESC-derived cells were also observed in Rosenthal's canal, close to surviving

endogenous SGNs, although their numbers were extremely low, indicating that delivery into the scala tympani was not an efficient route to direct exogenous cells to the Rosenthal's canal. Sekiya *et al*^[48] transplanted mESCs that had been exposed to the neuralizing activity of stromal cell-derived medium into the internal auditory meatal portion of the auditory nerve, aiming at minimizing the risk of damage to the cochlea and optimizing delivery to the target site. The group observed migration of the implanted cells along the damaged auditory nerve, into the Rosenthal's canal and to the scala media. Interestingly, no significant migration was observed when mESC-NPs were implanted in intact auditory nerves; instead, the cells extended numerous neuritic processes along the nerve. These observations, together with the fact that implanted cells exhibited varying morphologies depending on their location, pointed at an interaction of the exogenous cells with local environmental cues. In another series of experiments, Okano *et al*^[49] recorded higher rates of exogenous cell survival when implanting mESC-NPs in the modiolus of deafened guinea pigs, compared to their implantation in non-injured ears. Surviving cells differentiated to neurons that extended projections towards peripheral and central auditory targets. Interestingly, although synapse formation could not be demonstrated, some functional recovery was observed in some animals.

Work carried out by Albert Edge's group on hESCs identified BMP4 as a critical molecule to differentiate hESCs towards SGNs^[45]. When implanted in an *in vivo* gerbil model, hESC-derived NPs differentiated and engrafted in the auditory nerve trunk. The neurons extended projections to the sensory cochlear epithelium and towards the brain stem. Unfortunately, synapse formation could not be demonstrated. As mentioned above, hESC-NPs were also generated in Rivolta's laboratory (otic neural progenitors, ONPs)^[13]. ONPs were transplanted into the modiolus of ouabain-treated gerbils; implanted cells survived and formed an ectopic ganglion in the modiolus, with neurons that extended neurite projections to the OC. At 10 wk post-implantation some of the hESC-derived neurons had migrated from the ectopic ganglion to the Rosenthal's canal and some cells were seen migrating towards the brainstem; SYNAPTOPHYSIN staining pointed to the establishment of synaptic connections of hESC-derived neurons with neurons in the cochlear nucleus. Importantly, functional tests carried out at 4 wk post-transplantation demonstrated an improvement in ABR thresholds of animals that had received ONPs; functional restoration correlated to the increase in neural density resulting from ONP transplantation. ONPs were also obtained from hiPSCs^[39] and could be differentiated to neurons expressing combinations of sensory neuron and other neuronal markers (β III-TUBULIN, BRN3A, NF200, NEUROD1, ISLET1). These neurons established active synapses in co-cultures with HC-like cells that were also generated from hiPSC cultures^[39].

Survival of NPs derived from miPSCs has also been demonstrated *in vivo*^[50], following transplantation into mouse cochleae; some of the surviving cells expressed the glutamatergic neuron marker VGLUT1 and were seen to project neurites towards cochlear HCs^[50]. Differently to the approaches described above, Ishikawa *et al*^[22] differentiated hiPSC-NPs to neurons *in vitro*, prior to their transplantation. Although the cultures contained a mixture of neuronal types and they were at various stages of maturation, around 95% of the cells expressed VGLUT1. The authors conducted parallel differentiation experiments on Matrigel-coated plates and on 3D collagen matrices, obtaining similar results. Implantation of 3D cultures into the scala tympani of normal hearing-competent guinea pigs demonstrated differentiation of hiPSC-NPs to glutamatergic neurons although there was a significant decline in the number of surviving exogenous cells during the first two weeks following transplantation. Based on the loss of Oct3/4 expression in differentiated cultures, the authors defended the safety of their approach, since one of the risks posed by the transplantation of undifferentiated cell types such as ESCs and iPSCs is their potential to give rise to tumours. Nevertheless, the risk of tumour formation by implanted cells cannot be completely eliminated, and thus efforts have also been made to obtain otic sensory cell-like cells from fully differentiated somatic cell types that may overcome this problem. In line with this argument, Durán-Alonso *et al*^[51] applied to cultures of human fibroblasts the direct conversion protocol described by Costa *et al*^[44]. Over-expression of the GPA combination of transcription factors induced the expression of HC markers *MYOSIN VIIA*, *BRN3C* and *ESPIN*. Despite good transduction rates and a strong increase in HC gene transcript expression, clear morphological changes and expression of a combination of HC proteins (*MYOSIN VIIA*, *ANNEXIN A4*, *ESPIN*) was only observed when transduced cells were cultured in SFM containing EGF and RA, as employed by Rivolta's group on hFASCs^[13,28]; however, cell polarization or formation of stereocilia-like protrusions were not observed. Transcriptomic analyses of these cultures indicated an enrichment of genes related to HC development and differentiation, together with genes involved in neuronal differentiation.

Organoids: Some recent work has resulted in the establishment of 3D inner ear organoid cultures^[52-56], where differentiation of ESCs and iPSCs is conducted under culture conditions that sequentially recreate the stages leading to the development of various inner ear cell types *in vivo*. Thus the cultures are exposed to combinations of factors that activate and inhibit key signalling pathways to ultimately render the step-wise formation of non-neural ectoderm, pre-placodal and otic placodal epithelia, and otic vesicle epithelium that ultimately gives rise to HCs, SCs and sensory neurons^[52,54,57]. Differentiating cultures are provided with added extracellular matrix proteins that support the self-organisation of the cells into biologically more relevant 3D cultures than those growing as monolayers on tissue culture plates. This arrangement yields clusters of HC-like cells that express an array of HC markers and exhibit basal-to-apical polarization, ESPIN-labelled hair bundles containing functional mechano-transduction channels, and a diversity of voltage-dependent currents. Neurons also emerge within these cultures that establish synaptic contacts with developing HCs^[52,57]. Most of the work that has been carried out to date on inner ear organoids has focused on dissecting the identities and the complex interactions of the signalling pathways that regulate inner ear development^[57-59], but the numerous advantages offered by these cultures, in terms of cellular complexity, cell phenotype maturation and numbers of induced cells make them ideal substrates for other important applications in the field^[60,61]. At present there are important shortcomings to the use of inner ear organoids as sources for HC transplantation into the cochlea. A major hurdle is the fact that the applied protocols yield vestibular HC types^[56,62]. Work is underway to elucidate what elements are missing in the current cultures that may yield cochlear HCs; Jeong *et al*^[55] have recently described a series of modifications to the original method that result in the generation of various HC types in organoid cultures, some expressing cochlear HC markers. On the other hand, inner ear organoids may already constitute valid substrates to investigate SGN development and initiate studies towards a possible application in approaches to SGN regeneration. Perny *et al*^[54] have modified initially published protocols, obtaining mESC-derived cultures that contain a large number of neurons that express a whole array of sensory otic neuron markers (GATA3, PROX1, ISLET1, p75, MAFB, PERIPHERIN) and display electrophysiological properties similar to those of SGNs. Very interestingly, characterization of the neurons present in these cultures indicates that these are not vestibular neurons but cochlear SGNs.

Summarizing what has been discussed above, a number of exogenous cell types have been evaluated for their potential to replace damaged cells in the adult mammalian cochlea, in a quest for a promising approach to hearing restoration. Some of these results are summarized in **Figure 2**.

GENERATION OF OTIC CELL TYPES FROM ENDOGENOUS OTIC PROGENITORS

Various populations of stem cell/progenitor cells have been described in the mammalian inner ear at embryonic and neonatal stages^[14,27,63-65]. Initial studies on inner ear progenitors were based on the identification of cells in the inner ear that could self-renew in culture and give rise to different otic cell lineages when induced to differentiate^[64,66,67]; otic progenitors have been identified in the vestibular sensory epithelium, the OC, the spiral ganglion and the stria vascularis^[27,64,66,67]. On the other hand, since no regeneration takes place at later developmental stages following damage to HCs and/or SGNs, it has been traditionally accepted that there is no stem cell reservoir in the adult mammalian cochlea. This belief is supported by the fact that the features that characterize the progenitor cell populations that are present in the early stages of cochlear development are not encountered in more mature tissues^[63,64,68]. Sphere-formation tests^[63,66] indicate that there is a steep decline in the numbers of putative cochlear stem cell-like cells during the first three weeks following birth^[63,64,68]; this has been associated to a decrease in the expression of stem and early otic markers like *Nestin*, *Musashi1*, *Otx2*, *Mcm2*, *Pax2*, *Islet1*^[64], and the up-regulation of genes such as *P27* in SCs from the OC of older organisms^[63]. Work by Azel Zine's group^[69] demonstrated widespread expression of the stem/progenitor cell markers *Gfap*, *ABCG2*, *SOX2* and *JAGGED1* in cochlear SCs of P3 mice, and *Nestin* expression, which was mostly localized to inner phalangeal and border cells. Besides changes in the levels of expression of some of these genes, the authors unveiled a shift in the expression of *Gfap* and *Abcg2* from the OC to SCs in the limbus area, and of *Jagged1* to limbus interdental and Hensen cells; interestingly, these are areas where adenoviral-mediated *Math1* overexpression has led to HC regeneration in adult cochleae, resulting in improved hearing function^[70,71]. These and other observations add support

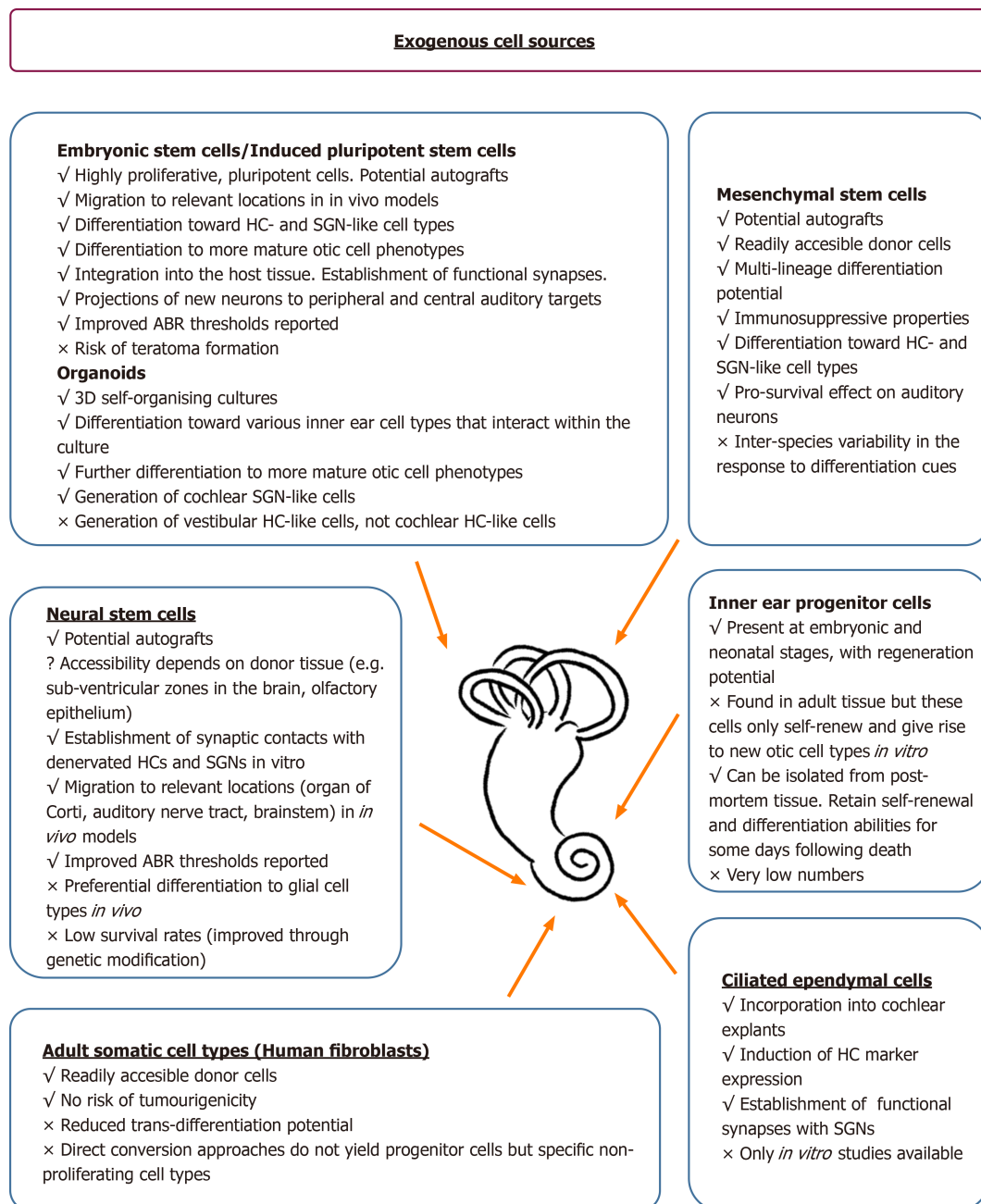


Figure 2 Overview of exogenous donor cell types that have been tested for their potential to give rise to hair cell- and / or spiral ganglion neuron-like cells. Advantages and disadvantages of the various donor cell types are indicated, as well as the main results that have been obtained with these cells in *in vivo* and *in vitro* studies. HC: Hair cell; SGNs: Spiral ganglion neurons.

to the notion that stem cell-like cells could still persist in the adult cochlea that have their regenerative potential curtailed by incoming signals from the surrounding tissue. Isolation from the post-mitotic cochlear epithelium of sphere-forming cells that can proliferate *in vitro* points at the presence of inhibitory signals originating from the cochlear tissue^[63,64,66]. Moreover, despite the inability of the adult cochlea to regenerate damaged cell types, stem cells have been isolated from fully mature cochlear tissues that self-renew and give rise to differentiated otic lineages *in vitro*^[15,16]. The numbers of these cells are very low; in addition, there are some technical difficulties associated to the study of cochlear tissues from adult mammalian models. As a result, the properties of various SC populations considered to be potential adult cochlear progenitor cells have been mostly explored on early post-natal animals, always bearing in mind that these models do not faithfully reproduce the conditions in the adult organ.

As already mentioned, a number of progenitor cells have been described in the embryonic cochlea^[14,65,72,73]. Chen *et al*^[27] isolated NESTIN+ SOX2+ PAX2+ otic progenitor cells from the cochleae of 10-week-old human foetuses; as discussed above,

these cultures could be propagated as adherent cultures and presented two clearly different cell morphologies, which could be differentiated towards HC or SGN lineages. Roccio *et al*^[14] later isolated a population of EPCAM+ CD311+ cells from the cochlea of 8-12-week-old fetuses; these cells proliferated in organoid cultures and could give rise to HCs. Other progenitor populations have been analysed at neonatal stages, soon after birth^[65,69,73,74]; this is the case of SOX2+ NESTIN+ cells of the great epithelial ridge, considered to give rise to new inner border cells and inner phalangeal cells when these are damaged during the first two weeks following birth^[73]; these progenitor cells are no longer present in the cochlea at the onset of hearing. A population of NESTIN+ cells that do not express HC or SC markers has been identified beneath the basilar membrane of post-natal animals^[72,75]. These cells, called tympanic border cells, proliferate over the first 1-2 wk following birth; then, their proliferative potential declines, correlating with decreased *Wnt* signalling. Jan *et al*^[72] observed migration of tympanic border cells into the OC and adjacent tissues during the first two weeks after birth; these cells then differentiated into HCs and various SC types. A small number of these cells are thought to remain in the adult cochlea, in a quiescent state. A good number of studies have been conducted in recent years to characterize a population of SCs that express the adult stem cell marker LGR5 (Leucine-rich repeat-containing G-protein coupled Receptor 5)^[65,74,76,77]. These have been identified as HC progenitors in the neonatal cochlea^[65,74,78], demonstrating some proliferative capacity and regenerating HCs in response to HC damage^[65,74], *Wnt* signalling activation^[65,79,80] and/or Notch signalling inhibition^[81,82]. Efforts are being made to further unveil the signalling cascades that regulate the proliferation and HC regeneration abilities of LGR5-expressing cells^[83-85], since expression of this stem cell marker persists in a reduced number of Deiter SCs in the adult cochlea^[76]. However, most data have only been obtained on neonatal animal models^[80-82,84]. The identification of a progenitor cell population in the cochlea and elucidating the reasons why these cells appear unable to regenerate the damaged tissue would be of the utmost importance; these progenitors would constitute an ideal cell source for regeneration therapies, placed at the appropriate location, and would override some of the main hurdles encountered by approaches that rely on the use of donor cells from exogenous sources (Figure 3).

CONCLUSION

While work is underway to better characterize the complex network of signalling cascades that regulate inner ear development *in vivo*, some promising results have been obtained when transplanting cells from exogenous tissues into the inner ear of animal models, demonstrating the formation of synaptic contacts between the implanted cells and HCs in the cochlear epithelium as well as with SGNs and neurons in areas of the brain stem^[13,48,49]. There are many important questions to consider when contemplating this type of approach. Sufficient numbers of transplanted cells must survive, differentiate and integrate both physically and functionally into the damaged inner ear tissue in order to enable some degree of hearing restoration. Regeneration of HCs in the OC will convey survival of the donor cells in potassium-rich endolymph, a hostile medium to a large number of cell types; work is underway to establish protocols that increase the chances of survival of implanted cells in the scala media without compromising the internal conditions that are required for the correct functioning of the organ^[86,87]. There is also the question of what cell type should be selected as donor cell and whether the cells should be transplanted as undifferentiated stem cells, partly differentiated progenitors, or fully differentiated cells. Stem cells and progenitor cells have been proposed as better candidates for transplantation than fully differentiated cells, on the argument that these cells should be more responsive to endogenous environmental cues. There are however concerns regarding their safety, in terms of a possible risk of tumour formation following implantation, and also the possibility that these cells may not differentiate to the desired cell type^[22]; this is one of the hurdles encountered by NSC transplantation, when differentiation towards glial cell types often overrides neuronal differentiation. Improved survival and differentiation rates have been observed when using donor cells genetically engineered to express lineage-specific genes^[7,30,42] and also when coupling exogenous cell transplantation with the administration of instructive factors^[24,30]. One of the reasons to pursue the trans-differentiation of fully differentiated somatic cell types is the risk of teratoma formation by transplanted cells, as reported by Nishimura *et al*^[88] following implantation of miPSC-NPs. A first report on the trans-differentiation of human fibroblasts to cells expressing HC markers constitutes a proof-of-principle study^[51]; future experiments should be conducted that allow the transient expression

Endogenous otic progenitor cells

✓ Ideal progenitor cell type placed at the appropriate location

✓ Differentiation to be guided by surrounding cochlear tissue

Is there a stem cell reservoir in the adult mammalian cochlea?

Stem cell-like cells isolated from embryonic and neonatal cochleae.

Very low numbers of stem cell-like cells in the adult cochlea, with some potential to self-renew and give rise to cochlear cell types *in vitro*

True identity of the progenitor cell population in the adult cochlea? Tympanic border cells? LGR5+ cells?

If there is a progenitor cell population, why does regeneration not take place?

Insufficient number of progenitor cells?

Inhibitory signals from the surrounding tissue?

Epigenetic changes in the progenitor cells?

Figure 3 Considerations on the presence of a putative progenitor cell population in the adult mammalian cochlea. There is yet no clear evidence that such cells are present in the adult cochlea. Some data have been obtained that support their existence; additionally, some reports point to changes in the epigenetic status of these progenitor cells and/or in the surrounding micro-environment as possible causes of their inability to activate a regenerative programme in the damaged tissue.

of the transgenes using non-integrative vectors. In addition, approaches such as that described by Itakura *et al*^[89], who have developed iPSC lines that carry an inducible caspase-9 gene, will ensure a safer application of these cells; this is all the more relevant when considering the vast potential of iPSCs as donor cells in instances where hearing loss is due to a genetic mutation. Survival and differentiation to appropriate cell lineages will also depend on the specific locations reached by the transplanted cells, which will in turn be affected by the route of implantation that is selected. Careful selection of the delivery route when considering donor cell-based therapies will be of paramount importance; thus, for example, although implantation into the scala tympani could be a method of choice when considering the convenience to the patient, this might result in insufficient numbers of exogenous cells reaching the target areas^[47,48]. It has been conclusively shown that, independently from the transplanted cell type, the survival and engraftment rates of the exogenous cells are much higher in areas of damage, probably due to the release of relevant factors into the local microenvironment. There are doubts as to whether the observations made on experimental animal models do in fact mimic the clinical situation, and whether transplanted cells would encounter a similar microenvironment in the patient. Identification of the factors that promote the survival and differentiation of transplanted cells in animal models will nevertheless constitute highly valuable information^[90]. There are other concerns to the transplantation of exogenous cells, such as the need for immunosuppression when receiving allogeneic donor cells; work by various groups have underlined the immunosuppressive properties of MSCs^[22].

Very importantly, a major obstacle to the integration of implanted cells in the damaged OC is its cellular complexity and the fact that this is a highly organized epithelium, refractory to the integration of exogenous cells. This and the fact that formation of ectopic ganglia by exogenous cell-derived neurons results in some recovery of the hearing function make HC replacement a longer-term goal than SGN regeneration, as supported by the promising results that some groups have reported^[13,49,91]. Yet, differentiation studies are proving highly valuable at dissecting the signalling pathways involved in inner ear development and their complex interactions. Huge progress has been made, as demonstrated by the generation of inner ear organoid models that are shedding very important information on inner ear development and provide excellent cellular models that may yet be used in future transplantation approaches. Moreover, the availability of inner ear organoids of human origin is of great importance, especially in light of the interspecies variability

that has been observed^[13,20]. Use of gene edited-hiPSC cell lines to generate organoids provides an extremely useful tool to identify instructive molecules and to study the effect of gene mutations on inner ear development^[57,59,61].

With regard to a re-activation of the regenerative program in the adult cochlea, although extremely interesting, this is still a largely unknown area. It has been demonstrated that cells with stem cell-like properties persist in the adult inner ear; characterization of the progenitor population in the vestibular system is proving less difficult, since this tissue maintains some very limited regenerative capacity in adult mammals^[92]. However, the identity of progenitor cells in the adult cochlea has not yet been confirmed. Data from the Raphael and the Zine labs^[69,71] suggest that there may be a reduced pool of stem cell-like cells in the limbus area of adult cochlea that have been displaced from their location in the OC during the neonatal period and that may be responsive to specific signals such as *Atoh1* expression. On the other hand, there are also low numbers of tympanic border cells and LGR5+ cells present in the adult cochlea; these populations are responsive to Wnt signalling and can give rise to HCs and SCs during postnatal stages. However, their role and properties in the adult cochlea have not been characterized. Very interestingly, new evidence is emerging that point at changes in the epigenetic landscape of LGR5+ cells in the adult cochlea, opening the door to new approaches to reactivate a regenerative programme in these cells^[93,94]. It is also important to remember that all the information we have on the various progenitor cell populations has been obtained on animal models. There are only two reports on the presence of LGR5+ cells in the human foetal cochlea^[14,95]. Intriguingly, Johnson Chacko *et al*^[95] have described LGR5 expression in cochlear HCs of 12-week-old fetuses, but not in SCs. Although there are doubts concerning the reliability of the currently available LGR5 antibodies, this observation raises the question of whether the data that have accumulated on murine cochlear LGR5+ cells are in agreement with the characteristics of their human counterparts.

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Recent advances of single-cell RNA sequencing technology in mesenchymal stem cell research

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stromal cells with great potential for clinical applications. However, little is known about their cell heterogeneity at a single-cell resolution, which severely impedes the development of MSC therapy. In this review, we focus on advances in the identification of novel surface markers and functional subpopulations of MSCs made by single-cell RNA sequencing and discuss their participation in the pathophysiology of stem cells and related diseases. The challenges and future directions of single-cell RNA sequencing in MSCs are also addressed in this review.

Key words: Mesenchymal stem cells; Single-cell RNA sequencing; Pathophysiology; Novel surface markers

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Core tip: Mesenchymal stem cells (MSCs) are an important kind of multipotent stroma cells *in vivo*. Previous studies have focused on large groups of cells. In this review, we focus on advances in the identification of novel surface markers and functional subpopulations of MSCs made by single-cell RNA sequencing and discuss their participation in the pathophysiology of stem cells and related diseases.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells with great potential for clinical applications^[1-3]. However, there are many problems that need to be solved before the wide application of MSC therapy. First, MSCs exhibit heterogeneity at multiple levels, for example, among donors and tissue sources and within cell populations^[4]. Although the criteria for MSCs were established in 2006 by the International Society of Cell Therapy^[5], they need to be improved on the basis of new knowledge. Surface markers for identifying specific groups of MSCs or predicting their potential for cell differentiation remain to be elucidated. MSCs are produced and expanded *in vitro* to assure the availability of sufficient cell numbers for clinical therapy. Cell morphology changes, proliferation ability decreases, along with other alterations in cell function, and how culture conditions influence MSCs remains unclear. Finally, the efficacy of MSC therapy varies among different clinical studies, and more data are needed to explore the mechanism of immunoregulation and tissue repair^[6]. Single-cell sequencing is a powerful tool for characterizing heterogeneous cell populations and identifying novel stem cell types^[7-13]. The aims of this review are to emphasize the advances in the identification of novel surface markers and functional subpopulations of MSCs by single-cell RNA sequencing (scRNA-seq) and discuss their participation in the pathophysiology of stem cells and related diseases.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells are defined as multipotent mesenchymal stromal cells that can be isolated from many adult organs. They were first reported in 1974 by Friedenstein^[14] and were described as colony-forming unit fibroblasts. These cells have the capacity to differentiate into mesodermal tissues, such as bone, cartilage, and fat cells^[15,16], as well as other tissues, such as myocytes and neural cells^[17]. Moreover, the trophic function of MSCs in supporting hematopoietic stem cells (HSCs) is well studied^[17]. In preclinical studies, the advantages of suppressing the inflammation and immunoregulation of MSCs have attracted great interest^[18,19]. On the basis of these properties, many clinical trials are using MSCs to treat orthopedic diseases, degenerative diseases, and autoimmune diseases affecting single or multiple organs.

CELL HETEROGENEITY OF MSCS

According to the minimal criteria developed by the International Society of Cell Therapy in 2006 for defining MSCs, they must be adherent cells with a spindle-shaped morphology in standard culture conditions; they must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules; and they must be capable of differentiating into osteoblasts, adipocytes, and chondroblasts *in vitro*^[5]. However, cell-to-cell variation exists at multiple levels (Figure 1). First, MSCs can be found in various kinds of connective tissues, even when cultured under standard conditions. MSCs from adipose tissue and bone marrow show different surface markers and differentiation capacities because of distinct biological, chemical, and mechanical stresses in stem cell niches^[20]. For MSCs originating from the same tissue, those from different donors present distinct functions due to the influence of age, health condition, and other individual differences^[4]. Moreover, MSCs form clones, and cell heterogeneity exists both interclonally and intracolonally. Kuznetsov *et al*^[21] reported that only half of clonal MSC implants differentiated into bone in mice. Extracellular matrix genes and osteogenic transcription factor-related genes show increases in highly osteogenic clones compared to poorly osteogenic clones^[22]. MSCs within a clone also present differences in cell morphology and differentiation ability. For instance, cells located at the outer periphery of clones express higher levels of genes (*MKI67* and *PODXL*) related to cell proliferation, while extracellular matrix genes (*VCAM1*) tend to exhibit higher expression in interior MSCs^[23].

To identify cell subsets with specific functions in heterogeneous MSCs, cell surface markers and global molecular signatures have been continuously explored^[24]. Single-cell-derived colonies with small, rapidly dividing cells show a high colony-forming efficiency. STRO-1, CD146, and CD271 have been identified as cell surface markers^[25,26]. However, markers of proliferation cannot predict the differentiation potential of MSCs, and cell subsets sharing similar surface markers exhibit different chondrogenic differentiation abilities under the same culture conditions^[27]. RNA sequencing and microarray analysis have helped to elucidate transcriptional

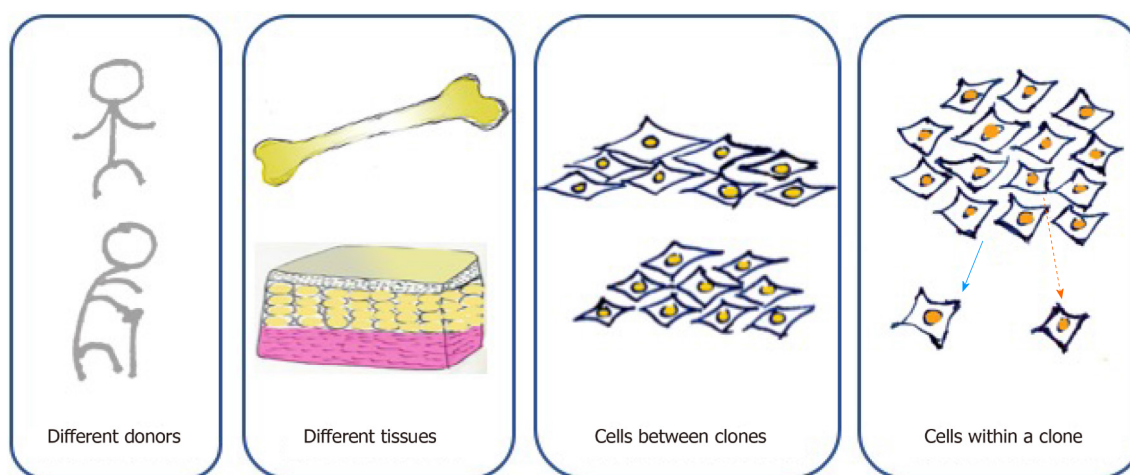


Figure 1 Cell heterogeneity of mesenchymal stem cells at multiple levels. Mesenchymal stem cells (MSCs) originating from different donors present distinct functions due to the influence of age, health condition, and other individual differences. MSCs originating from the same donor but different tissues, such as adipose tissue and bone marrow, show different surface markers and differentiation capacities because of distinct biological, chemical, and mechanical stresses in stem cell niches. Moreover, MSCs form clones, and cell heterogeneity exists both interclonally and intracloally.

signatures predicting differentiation potential. Osterix and distal-less homeobox5 are the main transcription factors involved in osteoblast differentiation^[28], while peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding protein alpha are a signature of adipogenic potential^[29]. In addition, MSCs with specific surface markers of differentiation potential may present various physiological functions. Studies have proved that CD105+ MSCs exhibit myogenic potential, which could help to repair the infarcted myocardium^[30]; however, the differentiation incidence is low *in vivo*. Other studies have shown that an enhanced paracrine effect could be more important in the myocardial repair of CD105+ MSCs, and the subpopulations need to be further identified^[31,32]. CD106 (VCAM-1) is a cell surface protein related to the adhesion of leukocytes to the vascular endothelium^[33]. CD106+ MSCs show enhanced multipotency, while their protein expression decreases with extended passaging or differentiation. Moreover, CD106+ MSCs display an immunosuppressive ability by mediating cell-to-cell contact with immune cells, as reported in human placenta^[34]. Increasing evidence shows that MSCs contain multiple subsets with specific surface markers. More work is needed to identify these subpopulations and clarify their biological functions.

SINGLE-CELL SEQUENCING

Cells are the smallest functional unit of organisms. Cellular heterogeneity is a universally acknowledged characteristic of biological tissues, including MSCs. Even in almost pure cell groups, gene expression differs among individual cells because of the variability of the intrinsic regulatory system and extrinsic microenvironment. Single-cell sequencing, which includes single-cell transcriptome-, epigenome-, and genome-sequencing technologies, provides powerful tools for characterizing heterogeneous cell populations^[10,13,35]. As scRNA-seq is one of the most widely used of these approaches, this review will mainly discuss the advances of scRNA-seq applications.

Tang *et al.*^[36] first reported the methodology for scRNA-seq in 2009, which provides an unbiased view of the transcriptome at a single-cell resolution. Since that time, scRNA-seq strategies have been constantly updated, including methods for cell capture, RNA capture, cDNA amplification, and library establishment and bioinformatics tools for bulk data analyses^[37,38]. For example, by using a combination of the one-bead-one-cell droplet approach and a special barcoding strategy, Drop-seq and inDrop systems were developed, which are high-throughput systems that can handle thousands or even tens of thousands of individual cells in a single experimental run^[39]. Defining subpopulations within a dataset of MSCs can be achieved by unbiased clustering and differential gene expression analysis^[40-42]. More recently, a “pseudotime” trajectory was produced in a reduced-dimension data space, which helps to record the spatial or lineage information of MSCs^[35,43]. To date, scRNA-seq has been widely applied in the fields of early embryo development, neuroscience, cancer, and stem cell research.

SINGLE-CELL SEQUENCING TO ASSESS THE MULTIPOTENCY FUNCTION OF MSCS

MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes. Specific surface markers and global molecular signatures have been reported in studies based on bulk cells or tissues^[4,44]. Novel functional subsets of MSCs have been identified at a single-cell resolution in recent years^[7,9,13,45]. Worthley *et al*^[45] identified a subpopulation of skeletal stem cells in mice that express gremlin 1 as osteochondroreticular stem cells in the bone marrow. This subset of cells can generate osteoblasts, chondrocytes, and reticular marrow stromal cells but not adipocytes. Chan *et al*^[46] reported skeletal stem cells (mSSCs) with a similar differentiation potential in mice. Subsequently, using scRNA-seq, Chan *et al*^[47] identified novel surface markers in human bone-derived cells that were similar to mSSCs. PDPN+CD146-CD73+CD164+ cells are likely enriched in human skeletal stem cells (hSSCs) in the bone. The self-renewal ability and multipotency of the cells are maintained, and injury-induced hSSCs (PDPN+CD146-CD73+CD164+) represent a regenerative response to skeletal injury in a human bone xenograft mouse model. In addition, hSSCs and hSSC-derived subsets express a series of potential hematopoiesis-supportive cytokines, including ANGPT1, CSF1, SDF, IL27, IL7, and SCF. Gene expression was also compared among hSSCs, mSSCs, and iPSC-derived hSSCs, and hSSCs isolated from any source were shown to cluster together. These cells are capable of forming the whole skeletal lineage hierarchy, which suggests great potential for use in regenerative medicine. Endosteal and outer periosteal compartments are both important to bone physiology, and each compartment maintains separate pools of cells separated by the bone cortex. Debnath *et al*^[48] identified periosteal stem cells (PSCs) in mice that form bone in an intramembranous manner, which differ from other skeletal MSCs that mediate endochondral ossification. In addition, the discrete existence of PSCs suggests that bone is composed of separate pools of stem cell progenitors that provide a special derived microenvironment for each type of stem cells (Table 1). To some extent, PSCs are specialized for intramembranous bone formation. The identified subsets contribute to fracture healing as well as the modeling of the bone cortex, providing a promising novel clinical target for bone fracture regeneration.

Tissue-resident mesenchymal progenitors (MPs) are responsible for tissue maintenance and regeneration. Scott *et al*^[9] revealed hypermethylated in cancer 1 (Hic1) to be a marker for MPs. scRNA-seq and ATAC-seq analysis demonstrated that Hic1+ MPs present distinct functions and lineage potential in skeletal muscle regeneration by providing stage-specific immunomodulation and trophic and mechanical support. Moreover, Giordani *et al*^[49] mapped ten mononuclear cell types in mouse muscle by scRNA-seq and illustrated gene signatures and key discriminating markers in each group, which can help to understand more about muscle-resident cell type identities and muscle diseases.

Regarding adipose tissues, Liu *et al*^[50] performed an analysis of adipose-derived mesenchymal stem cells (ADSCs) from three donors by scRNA-seq, and the resulting high-quality dataset is valuable for distinguishing the heterogeneity of cultured ADSCs at a single-cell resolution. Merrick *et al*^[51] found that dipeptidyl peptidase-4/CD26+ mesenchymal cells are highly proliferative, multipotent progenitors that give rise to ICAM1+ and CD142+ committed preadipocytes that can differentiate into mature adipocytes. The *in vivo* origin of adipose stem cells is currently poorly understood. Schwalie *et al*^[52] identified distinct subsets of adipose stem cells in the stromal vascular fraction of subcutaneous adipose tissue. The CD142+ group was shown to suppress adipocyte formation in a paracrine manner. The potentially key role of adipogenesis-regulatory cells in regulating adipose tissue plasticity is related to metabolic diseases such as type 2 diabetes.

Other studies have identified subpopulations of Col2a1-creER-marked neonatal chondrocytes that behave as transient mesenchymal precursor cells at the growth plate borderline^[53]. With the application of scRNA-seq technology, more subsets and specific surface markers of MSCs have been revealed, which helps not only to predict differentiation potential but also to explain the regulatory network under physiological and pathological conditions.

SINGLE-CELL SEQUENCING TO INVESTIGATE THE IMMUNOREGULATORY AND TROPHIC FUNCTIONS OF MSCS

MSCs can modulate both the innate and adaptive immune systems, including effects

Table 1 Characterization and function of stem cells

Ref.	Marker	Cell	Function	Species
Worthley <i>et al</i> ^[45]	Gremlin 1+	Osteochondroreticular stem cell	Generate osteoblasts, chondrocytes and reticular marrow stromal cells but not adipocytes	Mouse
Chan <i>et al</i> ^[46]	CD45-Ter119-Tie2-AlphaV+Thy-6C3-CD105-CD200+	Skeletal stem cells (mSSCs)	Bone cartilage and stromal progenitor	Mouse
Chan <i>et al</i> ^[47]	PDPN+CD146-CD73+CD164+	Skeletal stem cells (hSSCs)	Response to skeletal injury; express potential hematopoiesis-supportive cytokines	Human
Debnath <i>et al</i> ^[48]	CTSK-mGFP+CD200+CD105-	Periosteal stem cells (PSCs)	Form bone in an intramembranous manner	Mouse
Scott <i>et al</i> ^[9]	Hypermethylated in cancer 1 (Hic1) +	Mesenchymal progenitors (MPs)	Provide stage-specific immunomodulation and trophic and mechanical support	Mouse
Merrick <i>et al</i> ^[51]	Dipeptidyl peptidase-4 (DPP4)/CD26+	Mesenchymal cells	Give rise to ICAM1+ and CD142+ committed preadipocytes that can differentiate into mature adipocytes	Mouse
Schwalie <i>et al</i> ^[52]	CD142+	Adipose stem cells	Suppress adipocyte formation in a paracrine manner	Mouse

on neutrophils, macrophages, dendritic cells, natural killer cells, B lymphocytes, and T lymphocytes^[19]. For example, MSCs impede B lymphocytes from differentiating into plasma cells as well as secreting immunoglobulins. They can promote the generation of regulatory T cells while inhibiting the differentiation of helper T cells^[19]. The immunosuppression function can be executed *via* direct cell-cell interactions and paracrine actions. Many molecules secreted by MSCs are responsible for immunosuppression, including TGF- β , IL-10, PGE2, IDO, and NO. Although MSCs have been applied to treat several autoimmune diseases, such as Crohn's disease, rheumatoid arthritis, and systemic lupus erythematosus, the mechanism underlying the immunosuppressive ability of MSCs *in vivo* is not clear^[1,18].

In addition, MSCs are capable of supporting the maintenance, expansion, and differentiation of HSCs by producing growth factors, chemokines, interleukins, and extracellular matrix molecules. HSCs cotransplanted with MSCs *in vivo* ameliorated HSC engraftment and improved hematopoietic function recovery. In addition, MSCs secrete chemokines such as Ang-1 and CXCL12 to promote angiogenesis by recruiting endothelial progenitor cells. They can also produce neurotrophic factors that are important in neurogenesis and neurodegenerative diseases, such as amyotrophic lateral sclerosis and multiple sclerosis. The multipotency of MSCs is considered an important function for tissue regeneration and the treatment of degenerative diseases. However, less than 1% of transplanted MSCs could be found in the host bone of a patient who suffered from severe osteogenesis imperfecta. Similar observations were made in patients with eye diseases who were receiving MSC therapy, and no clear evidence showed MSC engraftment into the retina. Other functions, such as the roles of trophic factors, should also be considered in MSC therapy.

Although the importance of MSCs in bone marrow in supporting HSCs has been recognized since 1974^[14], the molecular complexity of this relationship and its response to stress are unclear. Tikhonova *et al*^[54] mapped the transcriptional signatures of bone marrow vascular, perivascular, and osteoblast cells in mice at single-cell resolution and revealed novel cellular subsets and cellular sources of pro-hematopoietic factors *in vivo*. The vascular notch delta-like ligands (Dll1,4) play critical roles in HSC differentiation and lineage commitment and are downregulated under stress-induced changes. These authors elucidated the cellular architecture of the bone marrow niche and revealed a heterogeneous molecular landscape that responds to stress. Severe *et al*^[24] reported that CD73+ BMSCs contribute to hematopoietic stem and progenitor cell engraftment and acute hematopoietic recovery.

Currently, effective cell surface markers are available to identify hematopoietic cells, and it is still difficult to elucidate the interactions with classical myeloid and

lymphoid cells *in vivo*. Jaitin *et al*^[55] analyzed spleen tissues using scRNA-seq, and single-cell transcriptional states in dendritic cells and hematopoietic cells were considered together to reveal gene-module activity and cell-type heterogeneity in both steady-state conditions and pathogen activation states.

SINGLE-CELL SEQUENCING TO INVESTIGATE CELL PROLIFERATION AND THE RESPONSE TO CULTURE CONDITIONS

Large numbers of MSCs are needed for clinical therapy, requiring cell expansion *ex vivo*. Although MSCs are capable of self-renewal and proliferation, these abilities decrease with time when the cells are cultured *in vitro* along with other functional changes^[56]. With cell cycle arrest and the loss of proliferation, some MSCs may undergo senescence^[56,57]. Oxidative stress and the dysregulation of regulatory factors associated with differentiation are related to the decreased differentiation potential of senescent MSCs. Bork *et al*^[58] revealed that DNA methylation identified at specific CpG sites is a typical epigenetic signature. When expanded *in vitro*, MSCs experience genomic DNA damage, and attempts to repair DNA damage seem to promote senescence, which could help defend against cell death. Other studies have shown that the functional decline associated with age can be reversed by manipulating epigenetic factors that are dysregulated. This could shed new light on the epigenetics of cell aging to improve therapeutic potential^[57].

Cells cultured *in vitro* are exposed to various conditions. The properties of the biomaterial interface, such as its topography, stiffness, and chemistry, can lead to transcriptional variations^[59-61]. Darnell *et al*^[62] found that the stiffness of hydrogels could regulate cytokine secretion by mouse mesenchymal stem cells. These authors further revealed many stiffness-sensitive genes by RNA-seq, which showed that stiffness can regulate the expression of MSC immunomodulatory markers. In addition, MSC signatures change as cell confluence increases *in vitro*, and 100% confluent MSCs may exhibit compromised pro-angiogenesis properties^[63]. Other studies are building upon the complexity of the niches produced *in vitro* to create a tissue-like system^[59]; however, there is a lack of research at a single-cell resolution regarding how cells sense and respond to the *ex vivo* culture microenvironment.

MSC THERAPY IN CLINICAL TRIALS

Lazarus *et al*^[64] reported the first use of MSCs as a cellular pharmaceutical agent in humans in 1995. Since that time, the number of clinical trials of MSCs has increased worldwide. The niche-like regenerative properties and anti-inflammatory ability of MSCs make them candidates for the treatment of acute tissue injury, chronic degenerative diseases, and inflammatory diseases^[4]. Conditional approval for MSC treatment in children with GvHD (graft-versus-host disease) was secured in 2012 in Canada, which was a historic event in the application of MSC therapy in the clinic. In addition, the use of MSCs to treat Crohn's-related enterocutaneous fistular disease was approved by the European Commission in 2018. Other high-quality clinical trials have focused on heart disease. Bartunek *et al*^[65] performed an MSC trial (NCT00810238) of heart failure secondary to ischemic cardiomyopathy in 2013, which showed improved cardiac outcomes. However, three years later, another phase 3 trial (NCT01768702) of the same MSC therapy for the treatment of chronic advanced ischemic heart failure was performed. No significant difference was found between the MSC-treated and placebo groups from baseline to 39 wk in the primary endpoint. The authors also evaluated ventricular remodeling at 52 wk. The data revealed an inverted U-shaped dose curve with worse outcomes at a higher dose delivery of MSCs^[66].

Although MSCs are effective for treating some cardiac patients, the underlying molecular mechanism is poorly understood. Lescroart *et al*^[67] revealed that the population of Mesp1 cardiovascular progenitors are the progenitors of distinct cell lineages and regions of the heart, identifying molecular characteristics in the early stage of mouse gastrulation. In addition to cardiomyocytes, interstitial cells, including fibroblasts and vascular and immune cells, are also important for heart repair. Farbehi *et al*^[68] identified more than 30 populations, representing nine cell lineages. The novel fibroblast lineage trajectory observed under both sham and myocardial infarction and in myofibroblasts leads to a uniquely activated cell state. To evaluate whether novel cardiovascular progenitors can differentiate into cardiomyocytes, scRNA-seq was

used, and laminin-221 was identified as the most likely cardiac laminin that was highly expressed during development. Moreover, cells coated with laminin-221 could be reproducibly differentiated at different time points^[69]. Future sc-RNA-seq studies will help to identify disease-related phenotypes and the transition trajectories of cells during disease development.

CONCLUSION

scRNA-seq has been applied and improved for more than ten years since 2009; it was highlighted as the “Method of the Year” in 2013^[70] and has recently become a routine approach for investigating cell heterogeneity. The challenges and limitations of the technology should also be considered. Eleven grand challenges in single-cell data science were reviewed by Lahnemann *et al*^[71]. For example, scRNA-seq data may show zero results, where a given gene has no unique molecular identifiers or reads mapping to it in a given cell. Such sparsity of scRNA-seq results can affect downstream analyses, which necessitates further methodological development. Regarding the application of MSCs, the lack of information other than transcript levels could lead to inaccurate analysis. Then, although novel cell surface markers and cell subpopulations may be identified by scRNA-seq, few interactions with surrounding cells and organisms can be demonstrated.

In the future, more efforts are needed to explore methods that provide multimodal data^[35]. Such methods may include the simultaneous profiling of multiple types of molecular data within a single cell, for instance, scRNA-seq coupled with DNA sequence or protein information. Other types of technologies may allow the detection and analysis of different kinds of data together. If the combination of multiple types of single-cell data across different experiments or experimental conditions can be achieved, we will obtain new information about MSCs from a transcriptome-centric perspective, which will undoubtedly improve the efficiency of MSC therapy.

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Energy metabolism in cancer stem cells

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Abstract

Normal cells mainly rely on oxidative phosphorylation as an effective energy source in the presence of oxygen. In contrast, most cancer cells use less efficient glycolysis to produce ATP and essential biomolecules. Cancer cells gain the characteristics of metabolic adaptation by reprogramming their metabolic mechanisms to meet the needs of rapid tumor growth. A subset of cancer cells with stem characteristics and the ability to regenerate exist throughout the tumor and are therefore called cancer stem cells (CSCs). New evidence indicates that CSCs have different metabolic phenotypes compared with differentiated cancer cells. CSCs can dynamically transform their metabolic state to favor glycolysis or oxidative metabolism. The mechanism of the metabolic plasticity of CSCs has not been fully elucidated, and existing evidence indicates that the metabolic phenotype of cancer cells is closely related to the tumor microenvironment. Targeting CSC metabolism may provide new and effective methods for the treatment of tumors. In this review, we summarize the metabolic characteristics of cancer cells and CSCs and the mechanisms of the metabolic interplay between the tumor microenvironment and CSCs, and discuss the clinical implications of targeting CSC metabolism.

Key words: Cancer stem cells; Differentiated cancer cells; Metabolic characteristics;

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Core tip: Accumulating evidence indicates that the inadequacy of many treatments is due to their failure to target cancer stem cells (CSCs). Therefore, CSCs are a promising target for cancer treatment. Recently, it has been reported that CSCs exhibit a unique metabolic phenotype compared to normal cancer cells (non-CSCs), and CSCs can dynamically transform their metabolic state to favor glycolysis or oxidative metabolism. However, the mechanism of the metabolic plasticity of CSCs has not been fully elucidated, and existing evidence indicates that the metabolic phenotype of cancer cells is closely related to the tumor microenvironment (TME). In this article, we summarize the metabolic characteristics of non-CSCs and CSCs, highlight the mechanisms by which CSCs alter their energy metabolism *via* interactions with the surrounding TME, and discuss the potential therapeutic strategies to target energy metabolism in CSCs.

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INTRODUCTION

According to the World Health Organization statistics, cancer is still the most common cause of death, although multiple therapy strategies have significantly improved the overall survival rate of cancer patients. Accumulating evidence indicates that the inadequacy of many treatments is due to their failure to target cancer stem cells (CSCs). CSCs widely exist in different types of tumors and possess the ability to form tumors. Therefore, CSCs are a promising target for cancer treatment. Unfortunately, there are currently few therapeutic options for CSCs because CSCs are resistant to conventional therapies^[1].

Recently, it has been reported that CSCs exhibit a unique metabolic phenotype compared to normal cancer cells (non-CSCs). Non-CSCs metabolize glucose to produce lactate through glycolysis even in the presence of sufficient oxygen^[2], which is now known as the Warburg effect. Unlike non-CSCs, CSCs may be highly glycolytic or oxidative phosphorylation (OXPHOS)-dependent depending on the niches where the CSCs are located. Targeting the metabolism of CSCs would be a new strategy for CSC treatment.

In this review, we summarize the metabolic characteristics of non-CSCs and CSCs, highlight the mechanisms by which CSCs alter their energy metabolism *via* interactions with the surrounding tumor microenvironment (TME), and discuss the potential therapeutic strategies to target energy metabolism in CSCs (Figure 1).

CHARACTERISTICS OF ENERGY METABOLISM IN NON-CSCS

Somatic cells obtain energy or ATP mainly through the tricarboxylic acid (TCA) cycle and OXPHOS in a normoxic environment. Unlike normal cells, non-CSCs are highly proliferating and produce ATP mainly by glycolysis even in the presence of sufficient oxygen supply, which is called the Warburg effect or aerobic glycolysis^[3,4]. The reason is that ATP produced by glycolysis is 100 times faster than that produced by OXPHOS^[5]. Meanwhile, cancer cells upregulate the expression of glucose transporters (GLUTs) to gain more glucose. Increasing studies have demonstrated that various GLUTs are upregulated in different types of tumors^[6]. This abnormal energy metabolism is a hallmark of cancer cells and is believed to be the root of tumor formation and growth^[7]. Several common mechanisms have been reported to be involved in the regulation of glycolysis in cancer cells.

The typical glycolytic pathway includes several reversible enzyme reactions and three irreversible reactions, which are known as the committed steps. The first committed step is the phosphorylation of glucose to glucose-6-phosphate, a process

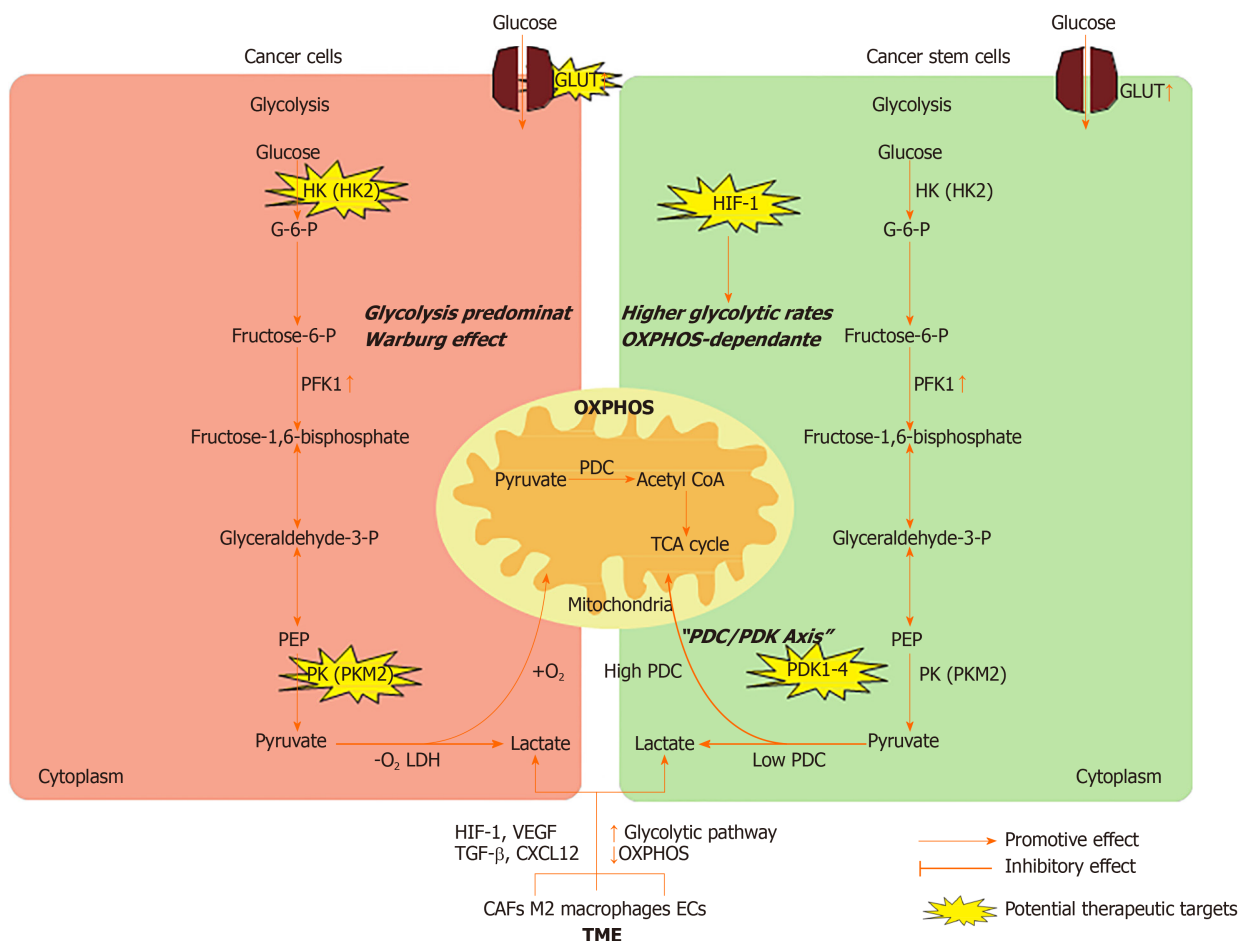


Figure 1 Energy metabolism in cancer cells and cancer stem cells. HK: Hexokinase; G-6-P: Glucose-6-phosphate; PFK1: 6-phosphofructo-1-kinase; PEP: Phosphoenolpyruvate; PK: Pyruvate kinases; PDC: Pyruvate dehydrogenase complex; LDH: Lactate dehydrogenase; PDK: Pyruvate dehydrogenase kinase family; TCA: Tricarboxylic acid; CAFs: Cancer associated fibroblasts; ECs: Endothelial cells; PDGF: Platelet-derived growth factor; HIF: Hypoxia inducible factor; TME: Tumor microenvironment; OXPHOS: Oxidative phosphorylation.

catalyzed by hexokinase (HK). There are four known HK isoforms in mammalian cells, and HK2 is expressed at high levels in cancer cells^[8]. The high expression and activity of HK2 in cancer cells have been exploited to detect and image tumors by a method known as [18F]-fluoro-2-deoxyglucose (FDG) positron emission tomography (PET). HK2 ablation reverses tumor formation *in vitro* and *in vivo* in non-small-cell lung cancer and breast cancer cells^[9]. Recently, HK2 was shown to be overexpressed in human colorectal cancer tissues and cell lines, and knockout of HK2 inhibited cell proliferation, colony formation, and xenograft tumor growth^[10]. The second committed step of glycolysis is catalyzed by 6-phosphofructo-1-kinase (PFK1), whereby fructose-6-phosphate (F6P) is converted to fructose-1,6-bisphosphate. Researchers have demonstrated that fructose-2,6-bisphosphate (F2,6BP) controls the rate of glycolysis by allosterically activating PFK1. F2,6BP is generated from F6P by the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/F2,6BPase or PFKB) family, which contains four isoforms. Generally, the kinase activity of the PFKFB3 isoform is increased in cancer, thereby increasing the intracellular concentration of F2,6BP and the allosteric activation capacity for PFK1^[11]. PFK1 is also elevated in cancer cells and controls the most important steps of glycolysis^[12]. The last committed step of glycolysis is catalyzed by pyruvate kinases (PKs), which transform phosphoenolpyruvate into pyruvate. There are four PK isoforms, and PKM2 is expressed in rapidly proliferating cells, including cancer cells^[13]. It has been reported that PKM2 was overexpressed in various malignancies, including lung, breast, prostate, blood, cervical, kidney, bladder, and colon cancers^[14,15]. During the last step of glycolysis, PK mediates the production of pyruvate. At this point, the gatekeeper enzyme pyruvate dehydrogenase complex (PDC) determines whether glucose metabolism ends in glycolysis or progresses to the TCA cycle and OXPHOS in the mitochondria. When energy production ends in glycolysis, lactate dehydrogenases (LDHs) convert pyruvate to lactate. Because LDHs are reversible, cells secrete lactate through monocarboxylic acid transporters (MCTs)

to promote the reaction and prevent a highly acidic environment. Currently, the expression of LDHs and MCTs has been observed in many tumors, and the effects of LDH and MCT inhibition in cancer cells are under investigation^[16]. When pyruvate enters mitochondria for OXPHOS, it is irreversibly converted to acetyl-CoA by PDC decarboxylation. Phosphorylation mediated by pyruvate dehydrogenase kinase enzymes inhibits PDC activity and is involved in the pathophysiology of many metabolically integrated diseases, including cancer^[17]. Several studies have shown that both the decrease in PDK activity and the enhancement of PDC activity through drug suppression or reduced expression are associated with reduced tumor growth *in vivo*^[18]. In gastric cancer, compared with that in normal tissue, the expression of PDC is lower in tumor tissue, which predicts a poor prognosis^[19].

Hypoxia and activation of oncogenes have been reported to be involved in the upregulation of glycolytic flux^[20]. Irregular perfusion and subsequent tissue hypoxia are common features of solid tumors. Under hypoxic conditions, overexpression of hypoxia-inducible factor-1 (HIF-1) upregulates and activates glycolytic proteins, such as GLUTs and glycolytic enzymes, to increase glucose absorption and phosphorylation^[21]. Meanwhile, a number of oncogenes, including *RAS*, *SRC*, and *Myc*, and activated AKT have been known to promote glycolysis by increasing the expression of GLUTs and glycolytic enzymes^[22-24]. In addition, mutations in tumor suppressors (*e.g.*, PTEN, p53, and VHL) are related to the acceleration of glycolytic flux in cancer cells^[25]. In prostate cancer, mutation of PTEN increases the translation of *HK2* mRNA by activating the AKT-mTORC1-4EBP1 axis, and deletion of P53 enhances the stability of *HK2* mRNA by inhibiting miR143 biogenesis^[26].

METABOLIC CHARACTERISTICS OF CSCS

The metabolic characteristics of CSCs have been the focus of attention. Numerous studies have shown that the metabolic characteristics of CSCs are highly heterogeneous. Unlike non-CSCs, which mainly utilize glycolysis, CSCs exhibit a glycolytic or OXPHOS-dependent metabolic phenotype^[27,28].

Evidence that glycolytic rate is higher in CSCs than in non-CSCs

Normal stem cells mainly use glycolysis to generate energy^[29]. Therefore, CSCs were hypothesized to mainly rely on glycolysis, similar to normal stem cells^[30]. Indeed, a series of studies in osteosarcoma, glioblastoma, breast cancer, lung cancer, ovarian cancer, and colon cancer have proven that CSCs are more glycolytic than non-CSCs, both *in vitro* and *in vivo*^[31-33]. Moreover, it has been observed that the glucose uptake, glycolytic enzyme expression, lactate production, and ATP content of CSCs are significantly increased compared with those of non-CSCs^[34]. Many genes, including *PFKFB4*, *PDK1*, and *PKM2*, are upregulated in brain CSCs^[35]. Inhibition of glycolysis or glucose deprivation can lead to the death of CSCs^[36,37].

The glycolytic switch in CSCs plays a key role in stemness rather than being a consequence of achieving pluripotency^[38]. Studies have demonstrated that inducing the metabolic transition from OXPHOS to glycolysis can increase CSC-like property of CD44⁺CD24^{low}EPCAM⁺ cells in basal-like breast cancer^[39]. Interestingly, HIF-1 has been identified as a central driver of the cascade of events that initiates CSC metabolic reprogramming from OXPHOS to glycolysis^[40]. Furthermore, the role of HIF-1 in tumors is related to stem cell characteristics, including self-renewal, pluripotency, tumorigenicity, and therapy resistance, as demonstrated in breast, hematologic, prostate, bladder, and central nervous system malignancies^[36,41,42]. In CSCs, HIF-1 alters glucose uptake and metabolism through upregulating GLUT expression, HK2 and PK activity during glycolysis, and LDHA levels at the end of glycolysis and downregulating pyruvate dehydrogenase (PDH) levels^[40]. Moreover, HIF-1 reduces mitochondrial reactive oxygen species (ROS) production by increasing the glycolytic pathway and decreasing the TCA cycle. Dynamic maintenance of ROS homeostasis is necessary to induce breast cancer stem cell phenotypes in response to hypoxia or cytotoxic chemotherapy^[43].

Evidence that CSCs obtain energy mainly from OXPHOS

On the other hand, some studies have shown that CSCs from multiple tumor types (*e.g.*, acute myeloid leukemia, glioblastoma, melanoma, and pancreatic cancer) rely on OXPHOS and have low glycolytic reserves^[44-47]. According to these studies, CSCs consume less glucose, produce less lactate, maintain higher ATP levels, and are more inclined to mitochondrial OXPHOS than their differentiated offspring. It is not ideal to study the metabolism of CSCs in an experimental environment lacking a relevant microenvironment. In the absence of better models that preserve the physiological state of CSCs, to keep the metabolic characteristics of CSCs intact, the best

experimental strategy is to isolate them directly from patients and analyze them immediately or in the first step of *in vitro* culture. In glioblastoma specimens, low-passage, patient-derived CSCs are more dependent on OXPHOS than their differentiated offspring^[48].

Although OXPHOS produces energy at a much lower rate than glycolysis, it is a much more efficient energy source. Moreover, CSCs also increase the utilization of extracellular metabolites, such as pyruvate, lactate, glutamine, glutamic acid, alanine, and ketone bodies, to adapt to OXPHOS metabolism^[49-51]. Similarly, OXPHOS-dependent CSCs may gain selective advantages in specific tumor microenvironments^[4]. In addition, studies have shown that elevated OXPHOS levels in CSCs can promote chemotherapeutic resistance. It has been demonstrated that Myc and MCL1 synergistically promote chemotherapy-resistant CSCs by increasing mitochondrial OXPHOS^[52]. Additionally, compared with non-CSCs, CSCs have higher mitochondrial mass and mitochondrial membrane potential ($\Delta\psi_m$), which reflects that CSCs are more prone to mitochondrial metabolism^[53-55]. Although the specific settings leading to the OXPHOS phenotype in all tumor types mentioned above have not been fully characterized, studies have shown that the mitochondrial biogenesis regulator and transcription coactivator peroxisome proliferator-activator 1 alpha (PGC1 α) may play an important role in maintaining stemness characteristics^[56]. In breast cancer, the inhibition of PGC1 α prevents mammosphere formation and CSC marker expression^[57]. In addition, in pancreatic CD133⁺ CSCs, PGC1 α is essential for OXPHOS function, self-renewal ability, and tumorigenesis^[54]. Growing evidence indicates that mitochondrial function is the basis for maintaining CSCs and can be a target for cancer treatment.

METABOLIC INTERPLAY BETWEEN THE TME AND CSCS

Tumor cells are located in the niche and constantly interact with the surrounding microenvironment. The TME is composed of extracellular matrix (ECM), cancer-associated fibroblasts (CAFs), macrophages, endothelial cells (ECs), immune cells, and a complex network of signaling molecules. It is well acknowledged that the metabolic phenotype of CSCs is regulated by the changing TME, and CSCs also remodel the metabolism of cells in the TME^[58].

Metabolic interplay between CAFs and CSCs

Recently, it was reported that CAFs could significantly promote the formation and growth of tumor spheres, which indicates an increased tumor formation potential^[59]. In addition, in pancreatic cancers, CAFs have been shown to induce epigenetic and metabolic changes in cancer cells and CSCs, thereby promoting tumor progression^[60]. CAFs also have metabolic adaptations, and these metabolic adaptations are considered to support glycolysis and play a key role in the use of nutrition by cancer cells and CSCs. Drivers of metabolic changes in CAF activation may include transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF), hypoxia, HIF-1 α , and ROS-mediated caveolin 1 inhibition^[61], which induce CAFs to switch from OXPHOS to aerobic glycolysis. After metabolic reprogramming, CAFs show enhanced catabolism and produce metabolites (lactate, glutamine, and ketones), which are used by cancer cells and CSCs to promote the production of oxidative energy and are associated with their potential for tumorigenicity and resistance to treatment^[62]. The metabolic interaction between cancer cells and CAFs in the TME is called the reverse Warburg effect^[63]. In the TME, cancer cells, CSCs, and CAFs express different types of lactate MCTs. Previous studies have suggested that epithelial cancer cells with a stem-like phenotype express MCT-1, while CAFs and other differentiated cancer cells express MCT-4^[64]. MCT-4-expressing CAFs and differentiated cancer cells secrete lactate through glycolysis, which is absorbed by epithelial CSCs that express MCT-1 and subsequently serves as a substrate for the TCA cycle^[63].

Moreover, the autocrine and paracrine effects of CAFs on cancer cells have now been fully studied^[65]. Compared with normal fibroblasts, CAFs have the ability to enhance the production of ECM components and secrete unique cytokines, including stromal cell-derived factor-1 (SDF-1)/C-X-C-motif chemokine 12 (CXCL12), vascular endothelial growth factor, PDGF, and hepatocyte growth factor^[66,67]. In breast cancer, CAFs promote the growth of breast cancer cells by secreting SDF-1^[67]. In addition, CAFs can secrete TGF- β and induce epithelial-mesenchymal transition (EMT) and eventually develop drug resistance^[66]. The induction of EMT is involved in the acquisition of stemness, leading to reduced mitochondrial metabolism and increased glycolytic flux^[68]. Many EMT regulators, such as TGF- β , Wnt, Snail, and distal-less homeobox-2 (Dlx-2), are involved in the metabolic reprogramming of cancer cells^[69].

Metabolic interplay between macrophages and CSCs

Tissue inflammation is an important part of the TME, and inflammatory cells and soluble mediators of inflammation, such as cytokines and chemokines, are abundant in the TME. Evidence suggests that inflammation has a dual role in tumor development^[70].

Macrophages are one of the most abundant components of the TME and play an active role in tumorigenesis. CSCs can attract macrophages into tumors by producing proinflammatory cytokines and chemokines^[71]. Once entering the tumor, macrophages are activated by factors such as IL-4 and transformed into tumor-associated macrophages (TAMs), which need to be metabolically adapted to survive the harsh tumor environment. The M1 polarization of macrophages is induced by endotoxin, interferon- γ , and interleukin (IL)-1 α , with a pro-inflammatory role while the M2 phenotype, induced by IL-4, IL-10, IL-13, TGF- β , and glucocorticoids, has anti-inflammatory effects and is involved in the resolution of inflammation^[72]. In the TME, TAMs are forced to undergo metabolic reprogramming to compete with cancer cells for nutrition. In solid tumors, hypoxia is one of the important factors determining the vascular structure of tumors. It was found that under hypoxic conditions, TAMs strongly upregulated the expression of the negative regulator of mTOR-DNA damage response 1 (REDD1)^[73]. REDD1-mediated mTOR inhibition can block glycolysis in TAMs and reduce their excessive angiogenic response, thereby forming abnormal blood vessels. Moreover, lactate secreted by tumor cells can stabilize HIF-1 α and induce the expression of vascular endothelial growth factor (VEGF) and M2 polarization of TAMs^[74]. Importantly, the metabolic effects of cancer cells on TAMs are not unidirectional. TAMs exposed to hypoxia or lactate secrete a variety of metabolic cytokines, including IL-6, tumor necrosis factor- α (TNF α), and CC-motif chemokine ligand 5^[75-77]. Polarized M2 TAMs secrete IL-6 to enhance 3-phosphoinositide-dependent protein kinase 1-mediated phosphoglycerate kinase 1 threonine (T) 243 phosphorylation, which promotes protein kinase 1-mediated phosphoglycerate kinase 1-catalyzed glycolysis^[77]. TAMs secrete TNF α to promote tumor cell glycolysis, with increased GLUT-1 and HK-2 protein expression^[75]. M2 TAMs stimulate CC-motif chemokine ligand 5 secretion, which increases cell migration, induces EMT in cancer cells, and promotes aerobic glycolysis in breast cancer cells *via* AMPK signaling^[76]. CSCs induce the M2 phenotype in TAMs, which secrete IL-6, IL-10, TGF- β , and EGF and drive CSC self-renewal by activating the STAT3/NF- κ B signaling pathway^[78].

Metabolic interplay between ECs and CSCs

Tumor cell growth and culture require multiple strategies to meet oxygen and metabolic needs, which involve the formation of new blood vessels. The angiogenesis process during tumor progression requires the recruitment of endothelial progenitor cells in the TME, during which endothelial progenitor cells differentiate into blood vessels^[79]. Angiogenesis is considered a key process for tumor progression and metastasis, and the TME and CSCs are considered to be important promoters of this phenomenon. Elevated lactate concentrations in the TME were found to affect EC signaling by enhancing IL-8/CXCL8 signaling, thereby promoting angiogenesis^[80]. In addition, CSCs can induce angiogenesis by secreting HIF-1, VEGFA, CXCL12, and other factors^[81]. Moreover, in recent studies, CSCs have been shown to differentiate into ECs in a process known as "vascular mimicry" and generate their own vascular system *via* a VEGF-dependent pathway^[81]. Recently, in a study of gliomas, researchers found that the injection of CSCs into the right frontal lobe of nude mice induced stronger angiogenesis and more hemorrhagic tumors than injection of non-CSC control cells. Meanwhile, the angiogenic advantage of the CSC counterpart may be supported by the 10-20-fold increase in VEGF secretion^[82]. Importantly, VEGF supports the angiogenic switch mainly by stimulating the glycolytic pathway. Studies have shown that when the glycolytic pathway is inhibited by the rate-limiting enzyme PFKFB3 in endothelial cells, the efficiency of angiogenesis is reduced^[83]. Other studies have found that under different environmental states such as hypoxia or altered glucose metabolism, CSCs can differentiate into functional ECs^[84].

The formation of new blood vessels and tumor angiogenesis are necessary conditions for tumor progression. Tumor blood vessels provide nutrition and oxygen for tumors and provide a pathway for tumor metastasis. Recent studies have shown that "vascular endothelial factors" released by ECs promote tumor progression^[85]. In brain tumors, endothelial cells interact directly with tumor cells and secrete factors that maintain these cells in a stem cell-like state^[86]. In head and neck squamous cell carcinoma, it is reported that 80% of CSCs are located near blood vessels. In addition, ECs secrete a variety of growth factors, including EGF, induce EMT through the PI3K/Akt signaling pathway, and promote the maintenance of CSC characteristics in head and neck squamous cell carcinoma^[87]. In breast cancer, ECs secrete TNF α ,

activate the NF- κ B signaling pathway in CSCs, and eventually develop chemical resistance to doxorubicin and cyclophosphamide^[88]. In colorectal cancer cells, ECs activate the NANOGP8 pathway associated with CSCs in a paracrine manner^[89].

CLINICAL IMPLICATIONS

Since CSCs have distinct metabolic phenotypes, which are a response to tumor progression and recurrence. On the other hand, the metabolic phenotype of CSCs is highly flexible between OXPHOS and glycolytic phenotype due to regulation of the TME. Traditional anticancer treatments aim to suppress rapidly proliferating cancer cells and fail to eradicate CSCs. Therefore, it is necessary to develop strategies to target metabolism of CSCs based on finding of the mechanisms involved in maintaining metabolic phenotype of CSCs. Recently, a large number of studies have been designed to selectively target the metabolism of CSCs.

Targeting glycolysis

Most CSCs satisfy their energy demands through glycolysis, which is subject to complex regulation. Therefore, various glycolytic enzymes or transporters can be targeted, such as GLUT1-4, HK, PDK1, and PKM2. A direct antiglycolytic strategy was proposed to block the glucose uptake *via* GLUTs, resulting in a complete disruption of energy metabolism. Previous studies have reported that several agents, such as phloretin, fasentin, and WZB117, have excellent anticancer effects through glucose uptake inhibition and energy deprivation in preclinical models^[90-93]. Phloretin is an antagonist of GLUT2^[90,91], which suppresses colorectal cancer cell growth by inducing cell cycle arrest and apoptosis *via* p53-mediated signaling^[90]. In addition, phloretin significantly inhibits the migration of cancer cells through paxillin/FAK, Src, alpha smooth muscle actin (α -SMA), and E-cadherin signaling^[91]. Fasentin is a GLUT1 inhibitor, which causes glucose deprivation and G₀-G₁ cell cycle arrest^[92]. As a selective GLUT1 inhibitor, WZB117 effectively inhibits glucose uptake, reduces the amount of intracellular ATP, and causes cell cycle arrest^[93]. Since GLUTs are widely expressed in normal cells, specific inhibition of glucose uptake in CSCs is challenging.

HK enzymes are responsible for glucose phosphorylation. Several HK inhibitors including 2-deoxy-D-glucose (2-DG), lonidamine (LN), genistein-27 (GEN-27), and benserazide, have been exploited for cancer treatment^[94-97]. 2-DG is a well-studied antiglycolytic agent, which competitively inhibits glucose transport^[98]. Several clinical trials of 2-DG activity have been designed in cancer patients^[96,99,100]. However, the anticancer efficacy and safety of 2-DG are still inconclusive. Currently, 2-DG is widely used in combination with other agents like cisplatin or docetaxel^[100,101]. Another HK inhibitor LN has completed preclinical studies and several clinical trials have explored its effects for cancer treatment^[102]. Unfortunately, no significant survival benefit of LN has been studied in several cancer types, such as lung, breast, and ovarian cancers^[97,103,104].

Pyruvate in the cytosol is converted into mitochondrial acetyl-CoA, which can enter the Krebs cycle *via* PDH enzymes. PDH is negatively regulated by the PDK enzyme, leading to a shift from OXPHOS to glycolytic metabolism. Thus, targeting PDK may be another attractive approach to inhibit cellular proliferation and cancer growth by inducing cancer cell or CSC metabolic reprogramming. Dichloroacetate (DCA) can activate mitochondrial PDH by inhibiting its regulator, PDK, and then enhance reprogramming of metabolism from glycolysis towards mitochondrial OXPHOS^[105]. Several clinical trials are ongoing for testing DCA efficacy as an anticancer agent^[106,107]. DCA is known to be relatively well tolerated with few significant side effects, and tumor response assessed by FDG-PET revealed stable disease in eight patients but no response in others^[107]. Overall, the current results of DCA for cancer therapy are preliminary, supporting a favorable toxicity profile but limited anticancer efficacy.

PK converts phosphoenolpyruvate into pyruvate by dephosphorylation to generate ATP. Kefas *et al.*^[108] demonstrated that PKM2 was expressed in glioma stem cells. Furthermore, PKM2 knockdown in glioma stem cells led to decreased cell proliferation and impaired metabolism accompanied by a reduced ATP level, which indicated that inhibition of PKM2 was a potential target in glioma stem cells^[108].

Moreover, CD44 was previously reported to interact with PKM2 and thereby enhance the glycolytic phenotype, suggesting that it could also become a preferential target. Tamada *et al.*^[109] reported that ablation of CD44 led to inhibition of glycolysis, with an increase in ROS, and enhanced the chemotherapeutic drug effect in glioma, colorectal cancer, or lung cancer cells. As a surface marker of CSCs, it can also be utilized for effective cytotoxic drug delivery^[110].

On the other hand, CSCs can rapidly transition their metabolic phenotype under

heterogeneous environmental conditions (such as hypoxia and glucose deprivation); thus, targeting adaptive mechanisms is also an optional strategy. As mentioned above, HIF-1 α is one of the principle factors that reprograms cells to undergo glycolysis instead of OXPHOS^[21,111] and is also involved in angiogenesis, metastasis, and cell survival^[112]. Hence, targeting HIF-1 α is a potential therapy for cancer treatment as well.

Targeting mitochondrial OXPHOS

Previously discussed evidence for OXPHOS in CSCs indicated mitochondrial metabolism to be a potential therapeutic target for eliminating CSCs. Inhibition of the OXPHOS pathway inhibits sphere formation and tumorigenesis, manifesting the vulnerability of CSCs to mitochondria-targeted drugs^[47,113]. Several pharmacological agents targeting OXPHOS are currently being explored in preclinical studies and clinical trials for cancer treatment.

The anti-diabetic agent metformin has emerged as a promising candidate for targeting oxidative metabolism in pancreatic CSCs^[54]. Several clinical results suggested that cancer patients with diabetes treated with metformin had a better prognosis than those treated with other antidiabetic regimens, which aroused researchers' interest in the mechanisms^[114,115]. Wheaton's study on metformin found that its antitumor activity involved the impairment of OXPHOS through direct inhibition of mitochondrial electron transport chain complex I^[116]. Nevertheless, the use of metformin for cancer therapy remains controversial. The first clinical trial testing the effect of metformin in pancreatic ductal adenocarcinoma patients did not report a positive outcome^[117]. The MYME trial failed to provide evidence in support of the efficacy of metformin in treating metastatic breast cancer patients^[118], while another study on non-small-cell lung cancer showed a significant change in outcome with the addition of metformin to standard chemotherapy^[119]. Phenformin, another biguanide formerly used in diabetes, can be delivered to mitochondria more efficiently than metformin^[4]. This agent also induces cancer cell death by inhibiting complex I and promotes apoptosis, offering promising preclinical results in certain cancer types^[120], but its clinical application remains to be studied.

Previous studies that were developed to screen compounds that selectively eliminate CSCs eventually discovered several drugs that inhibit mitochondrial activity. For example, the antibiotic salinomycin was identified and found to inhibit OXPHOS^[121]. The study showed that salinomycin treatment could result in reduced expression of breast CSC genes and thus inhibit mammary tumor growth *in vivo*. Another antibiotic, tigecycline, was also identified in a screen using OXPHOS-dependent leukemia cells^[122]. This agent was found to suppress OXPHOS by inhibiting mitochondrial translation associated with mitochondrial ribosomes. However, the clinical utility of these two drugs has not been fully elucidated.

As mentioned before, CSCs relying on OXPHOS show an elevated $\Delta\psi_m$, which can also be exploited for selectively increasing drug delivery to the mitochondria. Triphenylphosphonium, as a delocalized lipophilic cation, accumulates in the mitochondrial matrix and can be conjugated to small compounds for selective drug delivery to mitochondria^[123]. A recent study showed that conjugation of triphenylphosphonium to doxorubicin, a DNA topoisomerase II inhibitor, directed its activity towards mitochondrial DNA, promoting drug selectivity for cancer cells with reduced mitochondrial DNA integrity and preventing the acquisition of resistance by drug efflux^[124].

Due to the metabolic plasticity of CSCs, dual inhibition of the glycolytic and OXPHOS energy pathways may be the best approach against tumor growth. One study using such a strategy elegantly demonstrated that sarcoma cells are more sensitive than normal cells to synergistic effects of inhibiting glycolysis with 2-DG and OXPHOS with oligomycin or metformin^[125]. These results suggest that the dual inhibition of glycolytic and mitochondrial respiration may represent a better approach to eradicating CSCs and cancer treatment^[126,127]. Despite limited clinical evidence, targeting CSCs by blocking their metabolic components still holds great potential in improving cancer treatments. In practice, combinational treatments involving both a standard cytotoxic therapy and a CSC-targeted therapy will probably enhance the antitumor effect.

CONCLUSION

CSCs are thought to be the source of cancer cell production, resistant to treatment, and responsible for metastasis, and eliminating them could lead to a permanent cure for patients. Increasing evidence indicates that CSCs have distinct metabolic

phenotypes compared to most differentiated tumor cells. Therefore, the metabolic phenotype of CSCs has attracted great interest. CSCs have a unique metabolic phenotype and exhibit metabolic plasticity between high levels of glycolysis and OXPHOS, which may be related to the TME. However, the mechanisms of CSC metabolic plasticity still need to be clarified. Targeting CSC metabolism is considered a new treatment to eliminate cancer recurrence or metastasis. Combining CSC-targeted drugs with traditional anticancer treatments may be a more effective strategy for treating cancer. It is hypothesized that tumor stem cells may be derived from genetic changes in normal stem cells. It is necessary to accurately distinguish these two similar cell types to eliminate the damage that targeting CSCs may cause to normal stem cells.

A large number of studies have provided evidence that metabolic plasticity of CSCs exists in different malignancy, but the mechanisms involved in this process still need to be explored in the future. The role of metabolic reprogramming in CSCs in tumorigenesis, metastasis, drug resistance, and tumor relapse needs more evidence. Targeting the metabolism of CSCs is a promising approach in cancer treatment. However, it is necessary to identify specific targets to selectively inhibit the metabolism of CSCs without causing damage to normal stem cells. We expect that more preclinical and clinical studies will be carried out to find effective new anticancer agents.

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Human hair follicle-derived mesenchymal stem cells: Isolation, expansion, and differentiation

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Abstract

Hair follicles are easily accessible skin appendages that protect against cold and potential injuries. Hair follicles contain various pools of stem cells, such as epithelial, melanocyte, and mesenchymal stem cells (MSCs) that continuously self-renew, differentiate, regulate hair growth, and maintain skin homeostasis. Recently, MSCs derived from the dermal papilla or dermal sheath of the human hair follicle have received attention because of their accessibility and broad differentiation potential. In this review, we describe the applications of human hair follicle-derived MSCs (hHF-MSCs) in tissue engineering and regenerative medicine. We have described protocols for isolating hHF-MSCs from human hair follicles and their culture condition in detail. We also summarize strategies for maintaining hHF-MSCs in a highly proliferative but undifferentiated state after repeated *in vitro* passages, including supplementation of growth factors, 3D suspension culture technology, and 3D aggregates of MSCs. In addition, we report the potential of hHF-MSCs in obtaining induced smooth muscle cells and tissue-engineered blood vessels, regenerated hair follicles, induced red blood cells, and induced pluripotent stem cells. In summary, the abundance, convenient accessibility, and broad differentiation potential make hHF-MSCs an ideal seed cell source of regenerative medical and cell therapy.

Key words: Human hair follicle; Regenerative therapy; Mesenchymal stem cell; Tissue engineering; Cell differentiation

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Core tip: In this review, we describe the applications of human hair follicle-derived mesenchymal stem cells (hHF-MSCs) in tissue engineering and regenerative medicine.

the conduct of the study.

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We describe protocols for isolating hHF-MSCs from human hair follicles and their culture condition in detail. We also summarize strategies for maintaining hHF-MSCs in a highly proliferative but undifferentiated state after repeated *in vitro* passages, including supplementation of growth factors, 3D suspension culture technology, and 3D aggregates of MSCs. In addition, we report the potential of hHF-MSCs in obtaining induced smooth muscle cells and tissue-engineered blood vessels, regenerated hair follicles, induced red blood cells, and induced pluripotent stem cells.

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INTRODUCTION

The hair follicle is a skin appendage and dynamic mini-organ derived from tightly coordinated interactions between prototypic ectodermal-mesodermal cells early in embryogenesis. The hair follicle is easily accessible and contain stem cells from different developmental origins, such as epithelial stem cells, melanocyte stem cells, and mesenchymal stem cells (MSCs)^[1]. These stem cells continuously self-renew, differentiate, regulate hair follicle development, and contribute to hair follicle cycles which consist of the growth phase (anagen), regression phase (catagen), and rest phase (telogen) throughout adult life^[2]. During catagen and telogen, follicles prepare their stem cells for the next anagen. During anagen, bulge stem cells are activated by induction signals from the dermal papilla and migrate downward to the bulb region, where they proliferate and differentiate to regenerate the inner and outer root sheath, matrix, and hair shaft.

Many studies have focused on the epidermal stem cell lineage, which lies within the bulge region of the hair follicle, compared to MSCs derived from the dermal papilla or dermal sheath. In a pioneering study, Lako *et al*^[3] first demonstrated that dermal papilla and dermal sheath cells from transgenically marked donor mice could produce multiple lineages of the hematopoietic system in lethally irradiated mice, indicating the presence of multipotent stem cells in the dermal papilla and dermal sheath. Subsequent studies showed that dermal papilla or sheath cells from rat follicles expressed the cell-surface markers CD44, CD73, and CD90 as bone marrow MSCs and resembled bone marrow MSCs in their ability to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages^[4,5]. In 2006, the International Society for Cellular Therapy issued the minimal criteria for characterizing human MSCs. Specifically, cultured MSCs should be adherent fibroblast-like cells, express the surface markers CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and human leukocyte antigen-DR isotype. Furthermore, MSCs have osteogenic, adipogenic and chondrogenic differentiation potential *in vitro*^[6]. Therefore, dermal papilla or sheath cells from rat follicles may be a type of MSCs. A later study extended these findings to the human system and confirmed that dermal papilla or sheath cells from human hair follicles expressed the MSC immunophenotype and possessed multi-lineage differentiation potential; therefore, they were named human hair follicle-derived MSCs (hHF-MSCs)^[7]. Based on these previous studies, the hair follicle may be a readily accessible source of autologous human MSCs that can be used for tissue engineering and regenerative medicine.

Here, methods for isolating and expanding hHF-MSCs are presented and important recent advances in understanding the multi-potential of hHF-MSCs are summarized.

ISOLATION OF hHF-MSCs

When isolating hHF-MSCs, the first step is to obtain a complete hair follicle. When obtaining human tissues, studies should be conducted in accordance with the guidelines of the Helsinki declaration and appropriate ethical approvals should be in place. A frequently used method is to use collagenase type I to separate the intact hair

follicle from the human scalp skin^[8]. An intact hair follicle usually includes inner root sheath, outer root sheath, and connective tissue sheath (dermal sheath) as shown in **Figure 1**. The human scalp skin is usually obtained by skin biopsy from the scalp of a donor under sterile conditions^[7]. The obtained skin tissues are often full-thickness and therefore have an epidermis, dermis, and dermal white adipose tissue, as hair follicles are located in the adipose tissue. First, skin tissues are intensively rinsed with phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin solution, trimmed to remove underlying adipose tissues, cut into 2–4-mm small pieces, and digested with 1 mg/mL collagenase type I at 37 °C with occasional agitation. After 4 h of enzymatic dissociation, the epidermis can be peeled off from the dermis, and single-hair follicles are released from the dermis, filtered through a 40-mm cell strainer, and washed thoroughly with PBS to prevent contaminating the epidermal or dermal cells.

Another method is to obtain complete hair follicles by directly plucking them from the occipital region of the scalps, which eliminates invasive procedures associated with sampling^[9,10]. Hairs with intact follicles should be extensively washed with PBS containing 1% penicillin/streptomycin solution. The hair shafts are cut off, and the remaining hair follicles are manually transferred to the bottom of a 96-well plate, with one follicle per well, and cultured in 100 µL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 ng/mL basic fibroblast growth factor (bFGF) to allow for cell migration to the tissue culture plastic in a 37 °C/5% CO₂ incubator. Cells originating from the bulge region can be visually identified as epidermal keratinocytes, whereas cells migrating out of the dermal sheath or papilla show the morphological appearance of mesenchymal cells (fibroblast-like cells) (**Figure 2**). The wells populated with cells migrating from the dermal sheath or papilla are digested, pooled, and expanded under the same culture conditions. It should be noted that the cells migrating from the dermal papilla may contain a small number of neural crest stem cell-like cells. They can form neurospheres under serum-free culture conditions containing N-2, B-27, bFGF, and epidermal growth factor (EGF)^[11]. Li *et al.*^[12] found that the sphere-forming cells contained 1.14% ± 0.03% of dermal papilla cells. However, these neural crest stem cell-like cells need to be supplemented with ITS supplement and EGF when cultured *in vitro*^[13]. Therefore, they will gradually disappear with the increase of passage times under the hHF-MSCs culture conditions.

MSCs can also be separated from the dermal papilla or dermal sheath alone. Briefly, each strand of the hair follicle can be gently separated by microdissection away from the scalp tissues, ensuring that the dermal sheath and dermal papilla are intact. To isolate dermal sheath MSCs, the hair follicle is digested with 0.1% collagenase and 0.25% Dispase II at 37 °C for 30 min and then the dermal sheath is carefully separated from the main shaft of the hair follicle with a 30G needle under a dissecting microscope^[14]. After chopping the dermal sheath with scalpel blades, the samples are treated with 0.1% trypsin/0.02 M EDTA for 30 min at 37 °C. DMEM supplemented with 10% FBS and 10 ng/mL bFGF is used to quench the trypsinization process. After collecting the cell suspension from the dish into a centrifuge tube, the cell suspension is centrifuged at 200 × g for 5 min, aspirated to remove the supernatant, resuspended in an appropriate volume of the same medium, and expanded as hHF-MSCs.

As the dermal papilla is engulfed within the hair matrix, an inversion technique can be used to separate dermal papilla MSCs (DP-MSCs)^[4,15]. First, using a pair of scissors, the follicle through the matrix just above the papilla is transected to isolate the end bulb. Next, a fine needle can be used to invert the collagen capsule structure of the end bulb and expose the hair matrix and the dermal papilla residing inside. After removing the matrix component and any epithelial tissue still present from the papilla, the samples are cultured in DMEM supplemented with 20% FBS to allow for cell migration to the culture plate in a 37 °C/5% CO₂ incubator. Once the cells have proliferated to confluency, they can be passaged using standard cell culture techniques and the culture medium can be changed to DMEM supplemented with 10% FBS and 10 ng/mL bFGF.

EXPANSION OF hHF-MSCs

Currently, conventional cell culture techniques are still used to expand hHF-MSCs^[16]. Briefly, the isolated hHF-MSCs are seeded into a 100-mm cell culture dish and cultured in DMEM supplemented with 10% FBS and 10 ng/mL bFGF in a 37 °C/5% CO₂ incubator. The medium (10 mL for 100-mm plate) should be refreshed every 2 d. Once the cells are approximately 90% confluent, they should be sub-cultured using

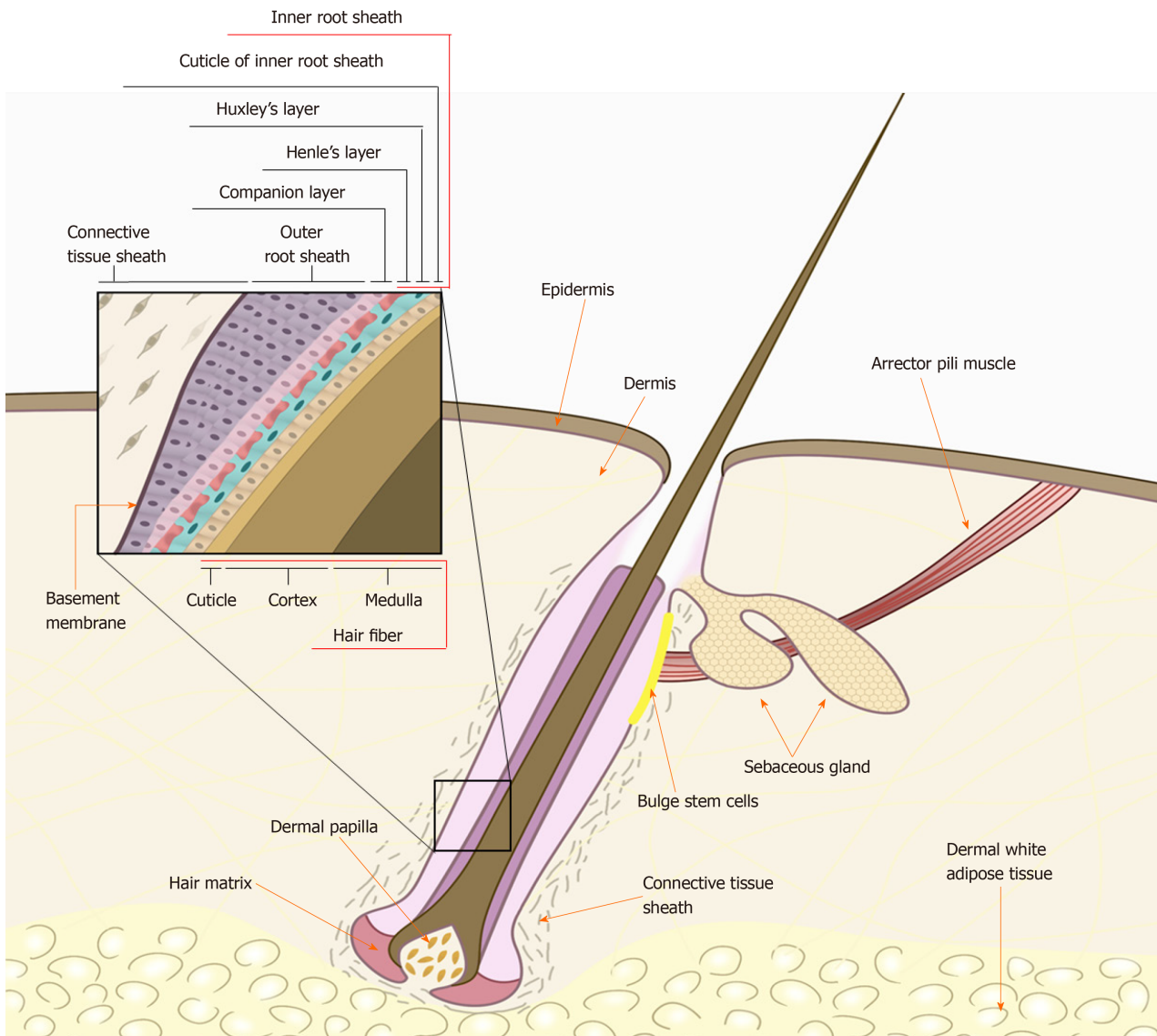


Figure 1 Schematic of the human hair follicle. An intact hair follicle usually includes the inner root sheath, outer root sheath, and connective tissue sheath (dermal sheath). The human hair follicle mesenchymal stem cells lie within the dermal papilla or dermal sheath (connective tissue sheath) of the hair follicle. Citation: Kiani MT, Higgins CA, Almquist BD. The Hair Follicle: An Underutilized Source of Cells and Materials for Regenerative Medicine. *ACS Biomater Sci Eng* 2018; 4: 1193-1207. Copyright© The Authors 2018. Published by American Chemical Society.

0.1% trypsin/0.02 M EDTA in a 100-mm cell culture dish.

For cell therapy and tissue engineering, a large number of hHF-MSCs with highly proliferative and multipotent differentiation potential are required. However, application of hHF-MSCs is restricted because they show replicative cell senescence and loss of multipotency in long-term *in vitro* culture^[17,18]. Bajpai *et al*^[19] found that hHF-MSCs could be maintained in culture for 11-12 passages (approximately 36 population doublings) before they started to show signs of cellular senescence. In addition to the 8-10 population doublings that occurred during the initial isolation and expansion stage, they may also undergo a total of 44-46 population doublings. It was estimated that a hair follicle could yield approximately 10^{15} hHF-MSCs before senescence occurred^[19]. It is important to develop effective strategies for maintaining hHF-MSCs in a highly proliferative but undifferentiated state after repeated *in vitro* passages. bFGF is a well-known growth factor that plays a critical role in the self-renewal, high proliferation, and multi-lineage differentiation potential of MSCs^[20-22]. Similarly, bFGF has been widely used in large-scale expansion of hHF-MSCs, particularly to prevent myogenic differentiation^[7]. Other growth factors, such as acidic FGF and EGF, have also been tested and shown to play a similar role in the expansion culture of hHF-MSCs^[23]. In recent years, some transcription factors have also been found to play a role in maintaining the proliferative capacity and multipotency of hHF-MSCs. Studies showed that ectopic expression of NANOG promotes cell proliferation and delays hHF-MSC senescence by upregulating PBX1 and activating



Figure 2 Isolation of human hair follicle mesenchymal stem cells. Human hair follicle mesenchymal stem cells migrating out of the dermal sheath or papilla show the morphological appearance of fibroblast-like cells. Bar: 200 μ m. Citation: Jiang Y, Liu F, Zou F, Zhang Y, Wang B, Zhang Y, Lian A, Han X, Liu Z, Liu X, Jin M, Wang D, Li G, Liu J. PBX homeobox 1 enhances hair follicle mesenchymal stem cell proliferation and reprogramming through activation of the AKT/glycogen synthase kinase signaling pathway and suppression of apoptosis. *Stem Cell Res Ther* 2019; 10: 268. Copyright© The Authors 2019. Published by Springer Nature.

AKT signaling^[16,24]. Lu *et al*^[25] also found that overexpression of OCT4 promoted the transcriptional activation of DNMTs, leading to elevated methylation of the p21 promoter, which promoted the proliferation and suppression of senescence of hHF-MSCs.

Moreover, compared to conventional 2D cell culture techniques, 3D-cultured MSCs show a higher yield in the same culture volume and stronger multipotency in large-scale generation of MSCs^[26,27]. Stirred-tank bioreactors with suspending microcarriers are the most widely used approach for the 3D culture of MSCs on a large scale. Stirred-tank bioreactors can make full use of the cultivation space and homogenized culture conditions, and enable process control, such as maintenance of pH and dissolved oxygen and medium supplementation^[28]. In addition, microcarriers offer a high surface area to volume ratio for the immobilization and expansion of adherent cells, and avoid the potential risks of tumorigenesis due to mutations caused by consecutive passaging^[29,30]. Our team utilized macroporous CultiSpher-G microbeads as microcarriers for the 3D culture of hHF-MSCs in stirred-tank bioreactors. The results revealed that hHF-MSCs quickly adhered to the microspheres and showed a 26-fold increase in the cumulative cell number after 12 d of expansion, with no significant difference in differentiation potential compared to 2D culture^[31].

In addition to microcarrier culture, 3D aggregate or spheroid culture without carrier and substrate provides enhanced cell-cell interactions and more accurately mimics the *in vivo* niche of MSC, which has been developed to expand MSCs^[32]. Previous studies demonstrated that 3D aggregates of MSCs exhibited higher proliferation efficiency, increased stemness and differentiative capacity, enhanced anti-inflammatory and angiogenic properties, and increased survival of transplanted cells compared to conventional 2D cell culture techniques^[33-35]. Recently, Topouzi *et al*^[15] and Higgins *et al*^[36] reported that 3D aggregates of DP-MSCs created by hanging drop cultures can restore the intact dermal papilla transcriptional signature and induce *de novo* hair follicles in non-hair-bearing human skin. However, few studies have evaluated 3D aggregates of hHF-MSCs, which remains a promising research direction.

DIFFERENTIATION OF hHF-MSCs

Similar to bone marrow MSCs, hHF-MSCs show adipogenic, osteogenic, and chondrogenic differentiation in the appropriate induction medium^[23,37]. Apart from their trilineage differentiation potential, accumulating evidence has demonstrated the potential therapeutic value of these cells in regenerative medicine by differentiating into smooth muscle cells (SMCs) and cell types of multiple different lineages. Here, we describe the differentiation potential of hHF-MSCs in detail.

Myogenic differentiation of hHF-MSCs

SMCs play a critical role in the occurrence and development of prevalent cardiovascular and respiratory diseases, such as atherosclerosis^[38] and asthma^[39], because of their contractile dysfunction. Emerging tissue engineering techniques offer

the possibility of reconstructing functional vessel walls by SMCs^[40]. Andrique *et al*^[41] used SMCs and endothelial cells to produce functional blood vessels with the correct configuration of lumen, which could also react to vasoconstrictor agents. hHF-MSCs have great potential to differentiate into SMCs. Using a tissue-specific promoter, a previous study showed that the smooth muscle alpha-actin promoter (P- α SMA) and fluorescence-activated cell sorting method can be used to isolate P- α SMA cells from hHF-MSCs^[7]. P- α SMA cells expressed specific markers of SMCs, including α SMA, calponin, and smooth muscle myosin heavy chain, and generated strong contractility in response to vasoactive agonists^[7]. The defining property of SMCs is their ability to generate contraction; thus, P- α SMA cells are considered as SMCs^[7]. Although this technique can achieve separation of a pure population of SMCs, it has the potential risk of foreign virus integration into the chromosome. Xu *et al*^[42] induced hHF-MSCs into contractile SMCs by stimulation with transforming growth factor β 1 and platelet-derived growth factor BB, which avoided the risk of effects from the lentiviral vector. In addition, Gao *et al*^[43] constructed tissue-engineered blood vessels using filled acellular umbilical arteries with hHF-MSCs under the regulation of transforming growth factor β 1, and the arterial grafts showed considerable vasoreactivity in response to humoral constrictors.

Application of hHF-MSCs in hair regeneration

hHF-MSCs are mainly located in the dermal papilla and dermal sheath of hair follicles and play an important role in regulating repeated hair follicle morphogenesis in adult life. A decrease in the number of hHF-MSCs per follicle can cause hair thinning and loss^[44]. Recently, Gentile *et al*^[45,46] used the medical device Rigenacons to develop autologous micro-grafts enriched in hHF-MSCs to treat androgenetic alopecia. The micro-grafts were obtained by centrifugation of a 2-mm punch biopsy of the scalp with the selection of a cell population with a diameter of 50 μ m. The mean hair density was increased significantly over baseline value after treatment with micro-grafts enriched of hHF-MSCs^[45]. Hair follicle morphogenesis is induced by tightly coordinated epithelial-mesenchymal interactions in the developing embryo. Similarly, bioengineered hair follicles can be prepared by the self-organization of epithelial and mesenchymal cells^[47]. In previous studies, murine hair follicle regeneration was achieved by intracutaneous transplantation of the bioengineered hair-follicle germ, which is generated by multicellular organization of follicle-derived epithelial stem cells and HF-MSCs in 3D stem cell culture. The bioengineered hair follicle exhibited similar tissue structures to the murine natural vibrissa follicle and grow pelage^[48]. These results suggest that hHF-MSCs are an important source of seed cells for human hair tissue engineering.

Hematopoietic differentiation potential of hHF-MSCs

Shortage of red blood cells caused by a lack of voluntary donations can threaten the lives of patients who require transfusion. hHF-MSCs may alleviate this dilemma because of their ability to differentiate into blood cells. A previous study showed that dermal papilla and dermal sheath cells generate hematopoietic colonies *in vitro*, and can contribute to multi-lineage hematopoietic reconstitution *in vivo* after transplantation into lethally irradiated recipient mice^[3]. Recently, Liu *et al*^[49] induced mature erythrocytes from hHF-MSCs by overexpressing OCT4 and hematopoietic cytokine exposure. This mature erythrocyte contained no nuclei, and expressed mainly the adult β -globin chain and rarely the fetal γ -globin chain. Numerous studies have shown that red blood cells produced from induced pluripotent stem cells (iPSCs) are generally incompletely enucleated and rarely express the adult β -globin chain, although iPSCs have been widely used to investigate treatments for diseases of the blood system^[50,51]. Therefore, hHF-MSCs may provide an alternative source of erythrocytes for potential autologous transfusion.

iPSCs from hHF-MSCs

iPSCs are a suitable seed cell source for regenerative medicine because their broad differentiation potential is similar to that of embryonic stem cells^[52]. iPSCs can be induced from somatic cells by ectopic expression of defined transcription factors^[53]. In a groundbreaking study, Wang *et al*^[10] successfully reprogrammed hHF-MSCs into iPSCs by lentiviral transduction with Yamanaka factors (OCT4, SOX2, C-MYC, and KLF4). These HF-MSC-derived iPSCs (HF-iPSCs) showed similar characteristics to embryonic stem cells in colony morphology, expression of alkaline phosphatase, and expression of specific human embryonic stem cells (hESCs) surface markers and endogenous pluripotent genes; additionally, HF-iPSCs formed teratomas containing representatives of all three germ layers after intramuscular injection into immunocompromised mice^[10]. HF-iPSCs have further expanded the application of hHF-MSCs in regenerative medicine. Bajpai *et al*^[54] derived contractile SMCs using

HF-iPSCs. Shi *et al*^[55] reprogrammed HF-iPSCs into functional hepatocytes expressing hepatic markers and drug metabolism-related genes. However, reprogramming of hHF-MSCs into iPSCs by lentiviral transduction with Yamanaka factors, although highly reproducible, is an inefficient method; therefore, identifying important transcription factors that can improve the efficiency of programming has become a research focus. Recently, our team found that co-transduction of PBX1 and Yamanaka factors into hHF-MSCs significantly improved the reprogramming efficiency by activating the AKT/GSK3 β signaling pathway^[56]. These results contribute to mass production of HF-iPSCs and further support the potential of hHF-MSCs in regenerative medicine.

CONCLUSION

hHF-MSCs have high proliferation ability and broad differentiation potential, and can be easily accessed by direct plucking of human hairs, showing numerous advantages over other cell sources in various clinical applications. We have described the tremendous capacities of hHF-MSCs in obtaining induced SMCs and tissue-engineered blood vessels, regenerated hair follicle, and induced red blood cells, as well as produced iPSCs in this review. However, hHF-MSC multipotency remains relatively unexplored as compared to the epidermal stem cell lineage in hair follicles or other human MSCs. The capacity for differentiation into other cell lineages as well as epigenetic modification during differentiation require further analysis. In addition, the role of hHF-MSCs in cell therapy has been investigated. hHF-MSCs transduced with the human hepatocyte growth factor (hHGF) gene can continuously secrete transgenic hHGF to promote liver cell regeneration and alleviate hepatic fibrosis^[57]. Engineered hHF-MSCs transduced with the release-controlled insulin gene can release human insulin in response to rapamycin and exhibited dramatic functionality in reversing hyperglycemia with no formation of detectable tumors after engraftment into mice^[58]. In summary, hHF-MSCs are a promising source of cells for the rapidly emerging field of cell therapy and tissue engineering.

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Stem cell therapy for COVID-19 and other respiratory diseases: Global trends of clinical trials

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Abstract

Respiratory diseases, including coronavirus disease 2019 and chronic obstructive pulmonary disease (COPD), are leading causes of global fatality. There are no effective and curative treatments, but supportive care only. Cell therapy is a promising therapeutic strategy for refractory and unmanageable pulmonary illnesses, as proved by accumulating preclinical studies. Stem cells consist of totipotent, pluripotent, multipotent, and unipotent cells with the potential to differentiate into cell types requested for repair. Mesenchymal stromal cells, endothelial progenitor cells, peripheral blood stem cells, and lung progenitor cells have been applied to clinical trials. To date, the safety and feasibility of stem cell and extracellular vesicles administration have been confirmed by numerous phase I/II trials in patients with COPD, acute respiratory distress syndrome, bronchial dysplasia, idiopathic pulmonary fibrosis, pulmonary artery hypertension, and silicosis. Five routes and a series of doses have been tested for tolerance and advantages of different regimes. In this review, we systematically summarize the global trends for the cell therapy of common airway and lung diseases registered for clinical trials. The future directions for both new clinical trials and preclinical studies are discussed.

Key words: Pulmonary diseases; COVID-19; Cell therapy; Exosomes; Clinical trial

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Core tip: Preclinical studies demonstrate significant improvement of lung disorders by stem cells and extracellular vesicles. Completed clinical trials show cell-based therapies are safe and tolerant for acute and chronic respiratory diseases. Current challenges for cell therapy of pulmonary illnesses are long-term safety, efficacy, and personal

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INTRODUCTION

Respiratory diseases are a top-ranked cause of death toll worldwide^[1]. Acute and chronic lung diseases, including coronavirus disease 2019 (COVID-19)^[2], acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), bronchopulmonary dysplasia (BPD), pulmonary arterial hypertension (PAH), silicosis, sarcoidosis, extensively drug-resistant tuberculosis, chronic obstructive pulmonary diseases (COPD)^[3], and idiopathic pulmonary fibrosis (IPF), have high morbidity and mortality. Amidst the list, COPD is the third leading cause of global fatality. Rapidly accumulating evidence in preclinical models suggests that cell-based therapy may be a promising therapeutic strategy for lung injury repair^[4]. For example, mesenchymal stromal stem cells (MSCs) derived from the umbilical cord blood, bone marrow, adipose, placenta, and other tissues are tested by registered clinical trials. Stem cells are able to repair injured airways and lungs by modulating multiple biological processes of the immune response, alveolar fluid clearance, cell fate, and drug delivery through paracrine and autocrine mechanisms, predominantly *via* extracellular vesicles (*i.e.*, exosomes)^[5-7]. However, the safety and benefits of cell therapy for the airway and lung diseases are under investigation by increasingly registered clinical trials. To analyze the trend of the clinical trials globally for the cytotrophy of pulmonary diseases, we summarized completed and ongoing trials registered to four databases from 1997 to 2020. This review will provide a brief state-of-the-science overview of the clinical studies of the respiratory diseases using various stem cells and extracellular vesicles.

OVERVIEW

We searched four broadly recognized databases worldwide: (1) The Clinicaltrials.gov (CT, <https://clinicaltrials.gov/>); (2) The International Clinical Trials Registry Platform (ICTRP, <http://apps.who.int/trialsearch/>); (3) The European Union Clinical Trial Regulation (EUCTR, <https://www.clinicaltrialsregister.eu/>), and (4) The PubMed databases. The total hits were 329 trials composed of 96 (CT), 23 (ICTRP), 3 (EUCTR), and 21 (PubMed) in aforementioned four databases, respectively. Some trials are with a status of “withdrawn” or had a disease condition of “complications after transplantation of stem cells”. After combining the published and dual registered studies, there are 120 trials. The vast majority of these clinical trials (82%) are testing the safety of stem cells for feasibility, tolerance, and severe adverse events (54 trials for phase I, 23 for I/II, and 21 for II). Few trials (3%) are phase III (Figure 1A). The status of these trials shows (Figure 1B): “recruiting” (30%), “not yet recruiting” (26%), “unknown” (18%), “completed” (18%), and “others” (8%). The published trails (8 of 21) are listed in Table 1. The first trial was registered in 1997, and suddenly the number shoots up mainly due to COVID-19 (Figure 1C). Geologically, the trials are predominately registered by China (42%) and the USA (22%) (Figure 1D). Currently, the main purpose of the clinical trials is to test the safety of stem cell therapy except few moving to test effectiveness. A completed list and additional features of these clinical trials can be found in the Supplementary table.

SPECTRUM OF STEM CELLS FOR RESPIRATORY DISEASES

Stem cells include multipotent embryonic stem cells and progenitor cells. MSCs are used in most clinical trials, probably based on the fact of well-tolerated and free of serious adverse events^[8]. Another consideration is availability. MSCs can be easily collected from the bone marrow, adipose tissue, muscle, peripheral blood, umbilical cord blood, and placenta^[9]. Umbilical cord blood derived-MSCs (UCB-MSCs) were

Table 1 Characteristics of published clinical trials

ID	Condition	Cell type	Case	Phase	Duration	Result
NCT00683722	COPD	Progenitor cells	62	II	2 yr	No infusional toxicities, deaths, and SAE
NCT01110252	COPD/ Emphysema	BM-MSCs	4	NA	1 yr	No SAE; significant improvement in the quality of life and clinical conditions
NCT01306513	Emphysema	BM-MSCs	10	I	8 wk	No SAE; increased CD31 expression
NCT01775774	ARDS	BM-MSCs	9	I	8 wk	No infusional toxicities, no SAE
NCT02097641	ARDS	BM-MSCs	60	II	8 wk	No SAE; improve MV and PEEP
NCT00257413	PAH	EPCs	31	NA	12 wk	No SAE; increased MWD, PAP, PVR, and cardiac output
NCT00469027	PAH	EPCs	7	I	1 yr	Well tolerated; increased MWD
NCT02181712	BOS	MSC	9	I	4 wk	No SAE

COPD: Chronic obstructive pulmonary disease; ARDS: Acute respiratory distress syndrome; PAH: Pulmonary arterial hypertension; BOS: Bronchiolitis obliterans syndrome; MWD: Mean walked distance; SAE: Severe adverse events; MV: Minute ventilation; PEEP: Positive-end expiratory pressure; PAP: Pulmonary artery pressure; PVR: Pulmonary vascular resistance; NA: Not available.

used in 43 trials, bone marrow derived-mesenchymal stromal cells (BM-MSCs) in 24 trials, mesenchymal stem cells in 15 trials, and adipose-derived stem cells (AD-SCs) for 12 trials (Figure 2A). Some trials are testing endothelial progenitor cells (EPCs), peripheral blood stem cells, placental mesenchymal stem cells, adult human stem cells, bronchi stem cells, menstrual blood-derived stem cells, bronchial basal cells, heart muscle progenitor cells, and lung stem cells. UCB-MSCs possess the highest proliferation rate, greatest anti-inflammatory ability, and lowest rate of senescence among all stem cells^[10]. BM-MSCs and AD-SCs are the most popular autologous stem cells^[11]. A combination of two or more types of stem cells is a standard regime for these clinical trials for lung diseases.

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) may serve as paracrine for MSCs to rescue damaged cells^[12]. MSC-derived EVs replicate 70% of the beneficial effects of MSCs and carry a variety of bioactive factors, including signal molecules and growth factors, to recipient cells^[13]. MSC-derived EVs are beneficial to the recovery of lung diseases in animal models^[14]. Pre-clinical studies have demonstrated that MSC-derived EVs significantly reduce lung inflammation and restore the function of injured lungs. It could be partially attributable to the improvement in macrophage phagocytosis and bacterial killing^[15-17]. Subsequently, the safety and efficacy of MSC-derived EVs are being tested for both BPD and COVID-19 pneumonia. It seems safer to deliver MSC-derived EVs rather than MSCs.

ROUTES AND DOSAGES

In general, the dosage of stem cells ranged from 1×10^6 to 1×10^9 cells/kg for a series of delivery, or a total dose of 2×10^6 to 1.2×10^9 cells. The dose of EVs is either 2.0×10^8 nanovesicles daily for 5 d or one dose of 20 pmol phospholipid/kg body weight. Stem cells and EVs were delivered *via* five routes: Intravenous perfusion for 73 trials, intratracheal administration for 18 trials, subcutaneous injection for 3 trials, intranasal instillation for 3 trials, and pulmonary artery injection for 1 trial (Figure 2B). Intravenous delivery (61% of analyzed trials) is a systemic route for cell therapy. Stem cells could be easily trapped in the pulmonary microcirculatory system and home to injured lobes^[18]. In contrast, local delivery, including intratracheal, intranasal, and pulmonary artery administration is the second most used route. Local delivery possesses the advantages of prolonging the half-time of cells, increasing the utilization efficacy, and decreasing side effects to other organs (off-target effects). It could be better for local lung injury repair, particularly for epithelial injury. Systemic delivery

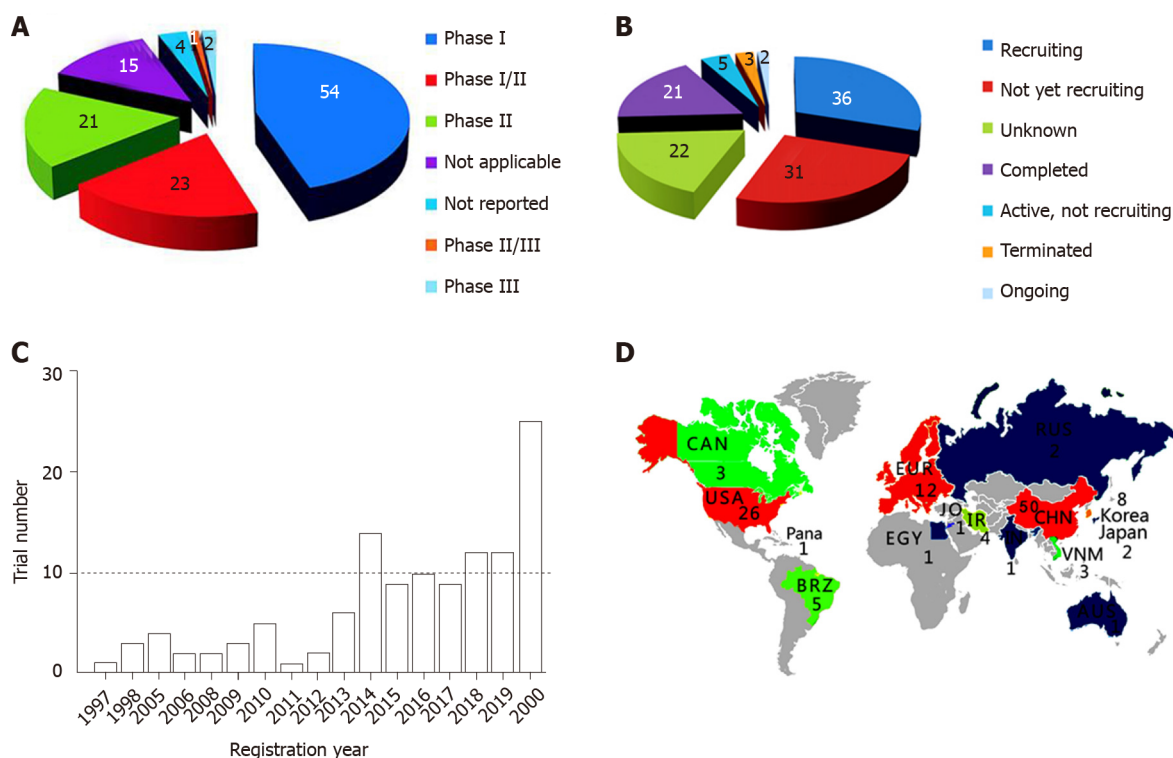


Figure 1 Characteristics of clinical trials. A: Clinical phases; B: Status of trials; C: Chronological distribution; D: Geographical locations. CAN: Canada; BRZ: Brazil; EUR: Europe; EGY: Egypt; IR: Iran; RUS: Russia; CHN: China; VNM: Vietnam; AUS: Australia; Pana: Panama; JO: Jordan.

of stem cells and EVs is applicable to systemic lung injury, including sepsis, multiple organ failure, or patients with severe pulmonary edema. A concern of local administration of stem cells and EVs is the distribution in both lungs. Based on the location of gastric acid aspiration injury and influenza animal models caused by the delivery of viruses intratracheally or intranasally, the impaired lobes are limited. In current trials, both local and systemic routes are used to BPD, pulmonary fibrosis, COPD, and silicosis. Local delivery of liposomal drugs and perfluorocarbon nanoparticles to the location of lung cancers is more effective than the systemic route^[19,20]. Therefore, the types, routes, and dosages of stem cells should be justified based on the personal conditions (precision/individual medicine)^[21].

RESPIRATORY CONDITIONS REGISTERED BY CLINICAL TRIALS

Clinical trials registered are designed to test the safety and benefits of stem cells for BPD 21 (18%), COVID-19 20 (17%), COPD/Emphysema 18 (15%), ALI/ARDS 12 (10%), pulmonary fibrosis 9 (8%), PAH 8 (7%). Few trials are for lung cancer, pneumoconiosis, silicosis, asthma, cystic fibrosis (Figure 2C). There is a significant increase since 2014 (Figure 1C), particularly after the outbreak of COVID-19.

COVID-19

COVID-19 coronavirus started in Wuhan, China, in December 2019 and has been spreading rapidly worldwide^[22]. There are no specific therapeutics yet for more than 1 million confirmed cases with 56-thousand deaths^[23]. Twenty-four registered clinical trials are designed to investigate the therapeutic effects of MSCs on COVID-19 (Supplementary table). MSCs have anti-inflammatory, anti-apoptotic, antimicrobial, and anti-fibrotic properties^[24,25]. COVID-19 has a higher risk of developing sepsis, multiple organ failure, including severe respiratory failure^[26,27], MSCs are assumed to have a beneficial effect for COVID-19, as supported by the promising results of a pilot study^[28]. A systemic review has recently summarized the ongoing trials regarding the cell therapy of COVID-19^[29]. We will, thus, not duplicate here.

ALI/ARDS

ALI is a common vital complication of systemic and pulmonary insults and developed as ARDS in the late stages^[30,31]. The fatality of ARDS is approximately 30 to 40%, and

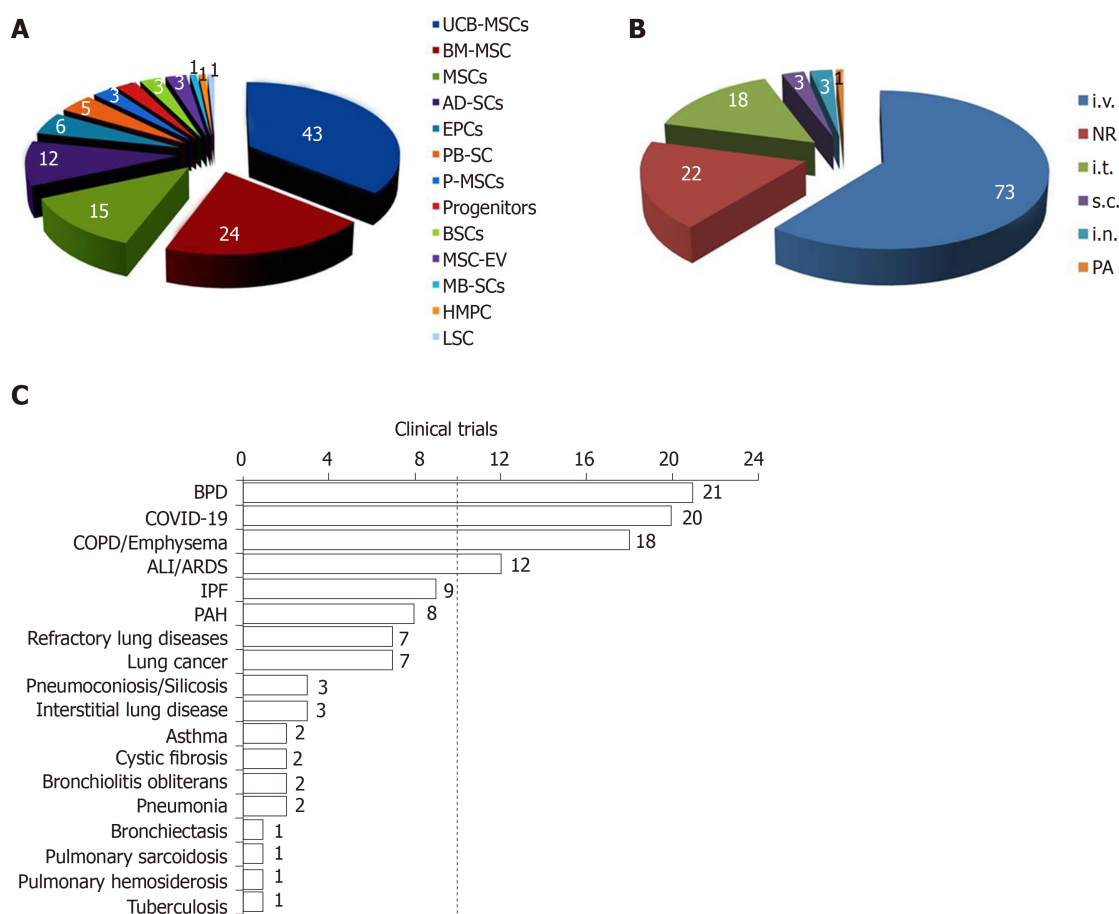


Figure 2 Respiratory diseases, types of stem cells, and administrative routes. A: Type of stem cells; B: Routes of delivery; C: Respiratory diseases. COVID-19: Coronavirus disease 2019; BPD: Bronchopulmonary dysplasia; COPD: Chronic obstructive pulmonary disease; ALI/ARDS: Acute lung injury/acute respiratory distress syndrome; PF: Pulmonary fibrosis; PAH: Pulmonary arterial hypertension; UCB-MSCs: Umbilical cord blood derived-mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; AD-SCs: Adipose-derived stem cells; MSCs: Mesenchymal stem cells; EPCs: Endothelial progenitor cells; PB-SC: Peripheral blood stem cells; P-MSCs: Placental mesenchymal stem cells; MSC-EV: MSC-derived extracellular vesicles; BSCs: Bronchial stem cells; MB-SCs: Menstrual blood-derived stem cells; HMPC: Human heart muscle progenitor cells; LSCs: Lung stem cells; i.v.: Intravenous; i.t.: Intratracheal; i.n.: Intranasal; PA: Pulmonary artery; s.c.: Subcutaneous; NR: Not reported.

even up to 49% in severe COVID-19 patients^[27], brings a serious economic burden globally^[32-34]. MSCs are promising, as shown in preclinical models of ARDS^[33]. A phase I trial has demonstrated tolerance and short-term safety (up to 6 months) of MSCs for ARDS patients^[35,36]. Further, a randomized phase IIa trial of ARDS treated by allogeneic mesenchymal stromal cells confirmed the safety of MSCs in 40 patients^[36]. These two completed clinical trials were registered in the United States (NCT01775774 and NCT02097641) (Table 1). In addition, the safety of MSCs for ARDS is being examined in a new phase II trial for ARDS^[37]. Besides MSCs, there are 12 clinical trials registered on the International Clinical Registration and five on the Chinese Clinical Trial Registrations for testing the MSCs from diverse resources. Although the benefits of cell therapy for ARDS are uncertain, the safety may not be a concern based on the results from completed trials.

COPD/Emphysema

COPD is characterized by tissue destruction, irreversible airflow limitation, caused by a combination of bronchitis and emphysema. COPD has high morbidity and mortality, which ranks the 3rd leading cause of deaths worldwide^[38]. Common drugs for COPD are corticosteroids and bronchodilators^[39]. MSCs are promising for COPD based on preclinical studies^[40]. There are 18 clinical trials registered to evaluate cell therapy in COPD or emphysema totally (Figure 2). Of these, only 3 have been completed (Table 1), two phase I and one phase II demonstrated the safety of cell therapy for COPD^[41-43]. Predominately, AD-MSCs and BM-MSCs are examined for COPD. Considering the limitations of small sample size and heterogeneity, a randomized, double-blind, placebo-controlled clinical trial is carried out in patients with COPD to follow up 2 years after MSCs infusion. Importantly, a phase I/II clinical study showed that four doses of UC-MSC treatment considerably alleviated the

severity of symptoms of COPD^[44]. Further studies are needed to confirm the effectiveness of MSCs, optimize the sources of MSCs, and select the best route to administer MSCs.

BPD

BPD is a chronic lung disease in premature infants, and usually causes various lifelong pulmonary complications (COPD and asthma)^[45,46]. The current treatment strategies of the BPD are unsatisfactory. The safety and efficacy of MSCs for earlier preclinical and clinical studies have been evaluated for BPD^[47,48]. Totally, 21 clinical trials are registered for cell therapy of BPD globally: China (8), Korea (7), United States (2), Spain (2), Canada (1), and Vietnam (1). Intratracheal infusion of allogeneic UCB-MSCs in preterm infants is safe and feasible^[49]. Inflammatory markers and growth factors in tracheal aspirate samples decrease after MSC transplantation^[50]. The same investigators have warranted a phase II clinical trial for intratracheal transplantation of UCB-MSCs to preterm infants with BPD (NCT01632475). The most of source of MSCs (90%) is UCB-MSCs in the 21 clinical trials, probably for UCB-MSCs are considered a better available source of MSCs than others^[51]. Given the small sample size of these trials, the interpretations of the safety shall be cautious, and it may be too earlier to draw conclusions for the benefits of cell therapy for BPD.

PAH

PAH is a progressive chronic disorder with high mortality and increasing prevalence, characterized by the remodeling of the pulmonary arteries and increased pulmonary infiltrates^[52]. Interventions with specific targets for PAH have been developed^[53]; however, the fatality is not reduced. Animal studies show that cell therapy may be the most potent approach for PAH^[54]. Therefore, 8 trials have been registered to date (Figure 2C). Amidst, 2 have been completed^[55,56]. EPCs are used in 8 clinical trials. Intravenous administration of autologous EPCs with or without gene editing of endothelial NO-synthase (eNOS) seems to be feasible and safe^[55,57]. A phase II trial of *eNOS* gene enhanced EPCs for PAH is ongoing (NCT03001414). Besides, the safety and effects of AD-MSCs on PAH is being tested. Due to the phase I/II trials are not a double-blind, placebo-controlled, the efficacy of EPCs for PAH is unknown. Taken together, the use of EPCs for formal clinical treatment requires a more rigorous review and experiment.

IPF

IPF is a chronic and irreversible interstitial lung disease characterized by diffuse alveolar inflammation and extracellular matrix remodeling^[58]. There are no effective regimes yet, but the administration of MSCs is evaluated as a new therapy^[59]. MSCs can prevent the progression of IPF in animal models^[60]. In this way, there are 9 registered clinical trials based on the benefits of cell therapy in preclinical studies. In a completed trail, a single dose of 2, 10, or 20 × 10⁷ cells/kg allogeneic BM-MSCs was intravenously delivered into 9 patients, whereas AD-MSCs were used. All three trails show the safety and well-tolerated of cell therapy and improved quality-of-life of IPF patients by MSCs^[61-63]. Of note, a standardized protocol is available for clinicians^[64]. Additional types of MSCs are tested in China, Australia, and Greece, including placental-derived MSCs and bronchial stem cells to compare the efficacy of them^[64,65]. Given that no severe adverse effects were observed during a period of 6-month follow-up, the safety and efficacy of intravenous infusion of autologous lung spheroid stem cells are recruiting patients.

Others

In addition to the aforementioned pulmonary diseases, clinical trials are registered for testing the safety and efficacy of stem cells for other refractory lung diseases, including lung cancer, silicosis, asthma, bronchiolitis obliterans, and tuberculosis (Figure 2C). Two clinical trials are recruiting bronchiolitis obliterans patients to evaluate the safety and feasibility of MSCs infusions. One phase I trial is evaluating the safety of allogeneic BM-MSCs (2-10 × 10⁷ cells/kg, i.v.) for asthma. The safety of intranasal delivery of MSC-trophic factor for asthma (NCT02192736) has initiated too. Autologous BM-MCs is further tested for silicosis (NCT01239862) based on a previous study^[66]. Radiation-induced lung injury is a new target of MSCs in the near future^[67].

CONCLUSION

In conclusion, there is a rapid pace of clinical trials on stem cell therapy for lung diseases in the last 5 years. Because of the heterogeneity of pulmonary diseases, a broad spectrum of stem/progenitor cells has chosen by registered trials. Meanwhile,

diverse routes for delivering and doses have applied based on both preclinical and clinical studies. It is a long-lasting debate if MSCs result in aggregating or clumping in the injured microcirculation and carry the risk of mutagenicity and oncogenicity. EVs could at least partially resolve these concerns. Mechanistically, the restoration of stem cell niches could be an innovative mechanism for cell therapy^[68,69]. To date, most of these trials are at an early stage for evaluating safety, feasibility, tolerance, and potential efficacy. Few clinical studies have described clinical improvements. Therefore, further optimization for cell therapy on respiratory diseases needs to be explored by more phase III and IV clinical trials. Cell therapy has significant challenges for gene editing stem cells, optimized route and dose, intervention regimes and applications for individual case, nevertheless, cell-therapy offers a most innovative strategy for unmanageable respiratory diseases.

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Multifaceted p21 in carcinogenesis, stemness of tumor and tumor therapy

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Abstract

Cancer cells possess metabolic properties that are different from those of benign cells. p21, encoded by *CDKN1A* gene, also named p21^{Cip1/WAF1}, was first identified as a cyclin-dependent kinase regulator that suppresses cell cycle G1/S phase and retinoblastoma protein phosphorylation. *CDKN1A* (p21) acts as the downstream target gene of *TP53* (p53), and its expression is induced by wild-type p53 and it is not associated with mutant p53. p21 has been characterized as a vital regulator that involves multiple cell functions, including G1/S cell cycle progression, cell growth, DNA damage, and cell stemness. In 1994, p21 was found as a tumor suppressor in brain, lung and colon cancer by targeting p53 and was associated with tumorigenesis and metastasis. Notably, p21 plays a significant role in tumor development through p53-dependent and p53-independent pathways. In addition, expression of p21 is closely related to the resting state or terminal differentiation of cells. p21 is also associated with cancer stem cells and acts as a biomarker for such cells. In cancer therapy, given the importance of p21 in regulating the G1/S and G2 check points, it is not surprising that p21 is implicated in response to many cancer treatments and p21 promotes the effect of oncolytic virotherapy.

Key words: p21; *CDKN1A*; Tumorigenesis; Circular RNA; Stemness of tumor; Cancer stem cells; Tumor therapy

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Core tip: p21, as a cyclin-dependent kinase regulator, suppresses cell cycle G1/S phase and retinoblastoma protein phosphorylation. As the downstream target gene of *TP53*, p21 expression is induced by wild-type p53. p21 was found as a tumor suppressor in several cancers by targeting p53 and was associated with tumorigenesis and metastasis.

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Notably, p21 is also associated with cancer stem cells. Moreover, p21 is closely related to cancer therapy, and it can promote antitumor effect of oncolytic virotherapy. These findings implicated multifaceted roles of p21 in cancer treatment.

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INTRODUCTION

The cell cycle is strictly regulated by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors to determine whether the cell is divided, quiescent or in the process of cell death in response to external stimuli and/or cellular microenvironment. Each type of cell has its own features during the cell cycle, and embryonic stem cells, germ cells and cancer cells rapidly divide. These cells also undergo cell cycle arrest and cell quiescence under adverse conditions. To harmonize the events, the cells have developed a sophisticated regulatory system. Malfunction of this mechanism results in serious disorders such as autoimmune disease and carcinogenesis.

p21 is encoded by *CDKN1A* gene, and was first identified as a CDK regulator that suppresses cell cycle G1/S phase and retinoblastoma protein (RB) phosphorylation. p21 is a major inhibitor of CDK2 and is therefore also known as CDKN1A (p21) or CDK-interaction protein (CIP)1^[1,2]. To be noted, p21 is a non-specific but commonly used name, and it has many aliases such as p21^{CIP1/WAF1} due to its multiple functions. In this context, p21 acts as the downstream target gene of *TP53* (p53), and its expression is induced by wild-type p53 and it is not associated with mutant p53. Hence, p21 is called wild-type p53 activating fragment 1 (waf1)^[3]. Since p21 was found as a potent inhibitor of G1 cyclin-dependent kinases in 1993^[4], it has been characterized as a vital regulator that involves multiple cell functions, including G1/S cell cycle progression, cell growth, DNA damage, and cell stemness. Early research revealed that G1/S cell cycle progression is negatively regulated by p21 binding to CDK and obstructing CDK interaction with its substrates^[5-7]. p21 inhibits tumor growth by targeting p53^[8]. Interaction between p21 and proliferating cell nuclear antigen maintains G2/M arrest after DNA damage^[8,9]. Importantly, the regulatory mechanism of p21 still attracts much attention in many fields.

P21 AND CARCINOGENESIS

Tumorigenesis is associated with imbalance between cell proliferation and cell death. p21, a CDK inhibitor, is related to cell cycle progression^[4]. In 1994, p21 was first found as a tumor suppressor in brain, lung, and colon cancer by targeting p53^[3]. Early research also revealed that the absence of p21 alters keratinocyte growth and differentiation and promotes ras-tumor progression^[10]. p21 is also associated with tumor migration and invasion. For example, cyclin D1 cooperates with p21 to regulate transforming growth factor (TGF)- β -mediated breast cancer cell migration and local tumor invasion^[11]. p21-activated kinase 4 regulates ovarian cancer cell proliferation, migration and invasion, and contributes to poor prognosis in patients^[12]. p21-activated kinase 1 stimulates colon cancer cell growth and migration/invasion *via* extracellular signal-regulated- and AKT-dependent pathways^[13]. In addition, environmental and microenvironmental factors also influence molecular mechanisms of carcinogenesis^[14], which may be related with variable p21 expression. These factors include diet, nutrition, lifestyle such as smoking or not, the living environment such as microbiome, and will influence the genome, epigenome, transcriptome, proteome, and metabolome of tumor and normal cells^[15]. Controversial aspects of p21 are decided by p21 location and p53 protein condition^[16]. p21 expression is induced by p53 under conditions of DNA damage or oxidative stress. For example, gambogic acid triggers DNA damage signaling that induces p53/p21Waf1/CIP1 activation through the ATR-checkpoint kinase 1 pathway^[17]. Notably, p21 plays a significant role in tumor development through p53-dependent and p53-independent pathways. For example, the cytoprotective aminothiol WR1065 activates p21 (waf-1) and

downregulates cell cycle progression through a p53-dependent pathway^[18]. p53-independent induction of the p21 (waf1) pathway is preserved during tumor progression^[19].

p21 is regulated by the tumor suppressor gene p53 and plays a regulatory role in inhibiting tumorigenesis; therefore, it is naturally assumed that p21 is also an antioncogene. A lot of experimental evidence confirms this conjecture. *In vitro*, expression of p21 negatively affects the malignancy of many cancer cell lines (TETs, ATLL and skin tumor) by inhibiting growth and inducing apoptosis^[20-23]. *In vivo*, experiments have found that upregulation of p21 leads to the arrest and invasion of breast cancer cells^[24]. The proportion of leukemia stem cell precursors *in vivo* is decreased in mice lacking p21 expression^[25]. In renal cell carcinoma, a decrease in p21 expression is seen as an important factor in the poor survival of clinical outcomes^[26]. In addition, p21 as CDKN1A can regulate T cell activation and attract innate immune cells to activate immune regulation^[27]. In some p53-deleted cell lines, TGF- β , RB, Tax, thrombopoietin, suppressor of cytokine signaling 1, 1,25-dihydroxyvitamin D3 and other factors can regulate p21 expression to achieve inhibitory effects^[28-33].

Circular RNAs (circRNAs) are newly-identified noncoding RNAs that covalently link 3' and 5' ends to form a closed loop and possess high stability^[34]. circRNAs can regulate tumor progression through regulating p21 expression. For example, circ-ITCH inhibits bladder cancer progression by sponging miR-17/miR-224 and regulating p21 and phosphatase and tensin homolog expression^[35]. circRNA affects cell cycle progression by forming complexes with p21. For example, forkhead box O3 circRNA retards cell cycle progression by forming ternary complexes with p21 and CDK2^[36]. However, there is no report on whether circRNA is expressed in p21 gene. Thus, whether p21 expresses circRNA to regulate tumorigenesis and migration and invasion is of importance.

P21 ROLE IN STEMNESS OF TUMOR

Several studies have shown that expression of p21 is closely related to the resting state or terminal differentiation of tumor cells. Various studies have shown that p21 is a key factor for the maintenance of stem/progenitor cells^[27,37,38]. Upregulation of p21 mRNA can inhibit proliferation of progenitor cells^[29]. Under normal steady-state conditions, abundant p21 expression is detected in both stationary hematopoietic stem cells and terminally differentiated mature blood cells. Knockdown of p21 results in proliferation of hematopoietic stem cells^[39,40]. Therefore, keeping the stem/progenitor cells at rest is crucial to prevent their premature depletion. In the bone marrow, p21 expression produces different results. In colony-formation experiments, p21 can promote the colony formation, proliferation and differentiation of murine bone marrow progenitor cells^[41]. Transient overexpression of p21 can lead to the development and differentiation of mononuclear/macrophages^[27,30]. The expression of p21 mRNA is increased over time in granulocytes, macrophages, megakaryocytes, and erythroblasts^[42]. The accumulation of its protein directly leads to final differentiation of cells^[43].

It is also reported that p21 is associated with cancer stem cells (CSCs). p21^{CIP1} attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and CSC-like gene expression *in vivo*^[44]. Bone morphogenetic protein7 regulates dormancy and recurrence of prostate CSC in bone via the p38/NDRG1/p21 signaling axis^[45]. Novel function of p21-activated kinase 3 in regulating Akt phosphorylation and pancreatic CSC phenotypes^[46]. p21 itself acts as a biomarker for CSCs. Gallagher *et al.*^[47] reported that cancer stemness is suppressed by p21-regulating mRNA and miRNA signatures in recurrent ovarian cancer patient samples, and presented a p53-p21 cancer stemness signature model for ovarian cancer. In addition, some researches have pointed out that miRNAs affect the stemness of CSCs by regulating p21. For example, miR-7 inhibits the stemness of prostate cancer stem-like cells and tumorigenesis by repressing the KLF4/PI3K/Akt/p21 pathway^[48]. miR-146b-5p overexpression attenuates stemness and radioresistance of glioma stem cells by targeting the HuR/lincRNA-p21/ β -catenin pathway^[49]. Thus, these studies support that p21 may play important roles in tumor stemness.

P21 AND TUMOR THERAPY

Chemotherapy is one of the main approaches for treating tumors. Given the importance of p21 in regulating the G1/S and G2 check points, it is not surprising that p21 is implicated in response to many cancer treatments. An early study from Zhao *et*

al^[50] showed that p21 is required for non-small cell lung cancer sensitivity to gefitinib treatment. In addition, it was shown that p21 protects cells from cisplatin cytotoxicity^[51]. For example, the p21 CDK inhibitors enhances the cytotoxic effect of cisplatin in human ovarian carcinoma cells^[52]. However, exogenous expression of p21 exerts cell growth inhibition and enhances sensitivity to cisplatin in hepatoma cells^[53]. Thus, p21 has different effects on cisplatin sensitivity for various tumors. Simultaneously, p21-mediated cyclins can regulate the resistance of some chemotherapeutic drugs. For example, the mechanism by which elemene reverses drug resistance of lung cancer cells is regulated and controlled by CDK8/p21 pathways^[54].

Oncolytic virotherapy has become one of the most promising therapeutic strategies for solid malignancies. p21 promotes the effect of oncolytic virotherapy. For example, Flak *et al*^[55] found that p21 promotes oncolytic adenoviral activity in ovarian cancer and is a potential biomarker. Conversely, RNA-interference-mediated knockdown of p21 enhances antitumor cell activity of oncolytic adenoviruses^[56]. Recently, we found that knockdown of p21 mediated by lentivirus inhibited the antitumor effect of oncolytic vaccinia virus in breast cancer cells (unpublished data). These studies revealed that p21 has different effects in oncolytic virotherapy for various tumors. However, the effect of interaction between p21 and Newcastle disease virus, herpes simplex virus1, and reovirus is unclear in tumor therapy.

Recent molecular pathological epidemiology (MPE) of cancer has increasingly becomes as a promising transdisciplinary and interdisciplinary field^[57]. According to MPE of cancer, p21 is associated with an increased risk of breast cancer in Chinese women^[58]. Thus, MPE can better study the pathogenesis, especially complex multifactorial diseases, and carry out personalized prevention and treatment. Notably, with the development of MPE and artificial intelligence, MPE by the analyzing of artificial intelligence has guiding significance for how to choose chemotherapy drugs to therapy tumors^[14,59].

CONCLUSION

Research on p21 has grown rapidly over the past 24 years. p21 is associated with carcinogenesis, CSCs, chemotherapy and therapeutic effect of oncolytic adenovirus (Figure 1). p21 acts as a tumor suppressor and has oncogenic potential. It was demonstrated that p21 sustained expression and its cytoplasmic localization are related to its carcinogenicity and tumor heterogeneity, and reflects its dual function depending on cellular and environmental conditions. High expression of p21 in stem cells plays a key role in this characteristic. High expression of p21 in normal stem cells is a manifestation of cellular health, from which we can speculate that p21 can serve as a marker of stem cells to respond to the state of cells. p21 is also associated with CSCs. p21 attenuates Ras- and c-Myc-dependent tumor epithelial mesenchymal transition and CSC-like gene expression. p21 itself acts as a biomarker for CSCs. p21 plays an important role in CSCs. In addition, further studies should be focused on MPE and investigate cancer risk factors, microbiome, immunity, and molecular tissue biomarkers, which was related with molecular pathologies in normal or diseased tissue. In tumor therapy, given the importance of p21 in regulating the G1/S and G2 check points, it is not surprising that p21 is implicated in response to many cancer treatments. p21 plays an important role in chemotherapeutic drug resistance, and some drugs can affect resistance by regulating p21-mediated cyclins. p21 also plays an important role in treatment with oncolytic adenoviruses. In the study of p21, a network of related genes will be established to fully understand its mechanism and make more targeted treatments.

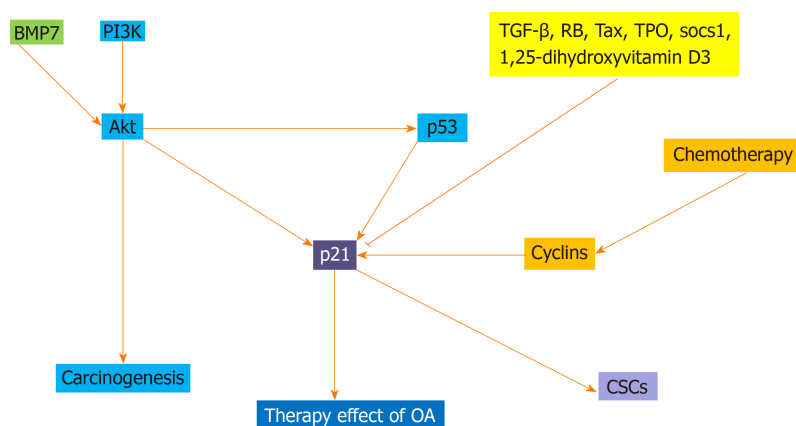


Figure 1 p21 is associated with carcinogenesis, cancer stem cells, chemotherapy and therapeutic effect of oncolytic adenovirus. TGF-β: Transforming growth factor-β; CSCs: Cancer stem cells; OA: Oncolytic adenovirus.

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Strategies for treating oesophageal diseases with stem cells

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Abstract

There is a wide range of oesophageal diseases, the most general of which are inflammation, injury and tumours, and treatment methods are constantly being developed and updated. With an increasingly comprehensive understanding of stem cells and their characteristics of multilineage differentiation, self-renewal and homing as well as the combination of stem cells with regenerative medicine, tissue engineering and gene therapy, stem cells are playing an important role in the treatment of a variety of diseases. Mesenchymal stem cells have many advantages and are most commonly applied; however, most of these applications have been in experimental studies, with few related clinical trials for comparison. Therefore, the methods, positive significance and limitations of stem cells in the treatment of oesophageal diseases remain incompletely understood. Thus, the purpose of this paper is to review the current literature and summarize the efficacy of stem cells in the treatment of oesophageal diseases, including oesophageal ulceration, acute radiation-induced oesophageal injury, corrosive oesophageal injury, oesophageal stricture formation after endoscopic submucosal dissection and oesophageal reconstruction, as well as gene therapy for oesophageal cancer.

Key words: Stem cells; Oesophageal diseases; Differentiative capacity; Regenerative medicine; Tissue engineering; Gene therapy

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Core tip: Stem cells have many characteristics and can be used to treat various diseases. Currently, the use of stem cells for the treatment of oesophageal diseases is developing but is still not very common. Therefore, this paper summarizes the relevant literature on stem cell therapy for oesophageal diseases, including oesophageal ulceration, acute radiation-induced oesophageal injury, corrosive oesophageal injury, oesophageal stricture formation after endoscopic submucosal dissection and oesophageal reconstruction, as well as gene therapy for oesophageal cancer, to promote better the development of stem cell therapy for oesophageal diseases.

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INTRODUCTION

There are a wide variety of oesophageal diseases, including mainly inflammation, tumours and injury. Treatment methods include drugs, endoscopic treatment and surgery^[1]. With the development of stem cell-related research and technology, stem cells have gained increasing attention in the context of treating various diseases^[2].

By definition, stem cells have the capacity for multilineage differentiation and self-renewal. They can differentiate into many specific types of cells *in vivo*^[3]. Stem cells can divide into totipotent, pluripotent, multipotent and unipotent stem cells according to their differentiation potential. Totipotent stem cells can differentiate into any kind of cells, and pluripotent stem cells can differentiate into cells of all three germ layers. Pluripotent stem cells can differentiate into many kinds of cells, but they may not cover all cells of one germ layer, and unipotent cells can only differentiate into one type of cell^[4-6]. Stem cells can be divided into embryonic stem cells and adult stem cells according to their source. Embryonic stem cells at the morula stage, which are a kind of totipotent stem cells, have the differentiation potential to form a complete individual. With embryonic growth, the potential of stem cells continues to decline from totipotency to pluripotency^[7]. Adult stem cells are undifferentiated cells existing in a kind of differentiated tissue that can self-renew and specialize to form cells that make up this type of tissue. Adult stem cells can include pluripotent stem cells and unipotent stem cells^[6]. For example, haematopoietic stem cells are the most characteristic pluripotent stem cells and can differentiate into at least 12 kinds of blood cells^[8]. Mesenchymal stem cells (MSCs) can differentiate into a variety of mesodermal cells (such as bone, cartilage, muscle, and fat cells) and other blastodermal cells (such as neurons)^[9].

Currently, MSCs are studied the most intensely and used the most widely. MSCs are relatively abundant and easy to isolate and culture. The main sources include adult bone marrow, umbilical cord or placental blood and adipose tissue, the latter of which is being increasingly developed and used as a source^[10]. MSCs can also be isolated from the periosteum, skeletal muscle, teeth and other tissues^[11,12]. Isolated MSCs can be used to treat tissue and organ damage and functional failure. MSCs have a powerful proliferation ability and multidirectional differentiation potential and can differentiate into osteoblasts, chondrocytes, adipocytes, liver cells, muscle cells, stromal cells and other cells under appropriate conditions *in vivo* or *in vitro*^[13]. Stem cells can also be used as carriers for gene therapies. In particular, MSCs are the most widely used for this purpose. They can not only be easily transfected with exogenous genes but can also express the protein of exogenous genes and retain their own phenotype after the introduction of exogenous genes^[14]. This characteristic, combined with the multilineage differentiation potential of MSCs, renders MSCs potentially ideal target cells for gene therapy. MSCs have low immunogenicity and can reduce the immune exclusion effect during cell transplantation^[15]. Currently, stem cells can be cultured and isolated artificially *in vitro* according to certain purposes, and various cells, tissues and organs can be constructed using stem cells as a source for transplantation. Stem cells also exhibit homing; that is, under the influence of many factors, stem cells will migrate in a directional manner^[16]. It is widely believed that the mechanism is based on the release of some factors from the site of injury, which bind to receptors for these factors on the surface of stem cells^[17]. This characteristic allows stem cells to serve as a carrier of many therapeutic agents (Figure 1).

Stem cells can be effective in the treatment of many diseases, such as cardiovascular diseases, nervous system diseases, bone and cartilage diseases and inflammatory diseases. However, stem cells are not commonly used in the treatment of the oesophageal diseases. The mechanism of using stem cells for the treatment of some common diseases of the oesophagus can be similar to that applied in the treatment of other diseases. Based on the characteristics of the oesophagus itself, stem cells could play an even greater role. The following describes the use of stem cells for the treatment of different oesophageal diseases (Table 1).

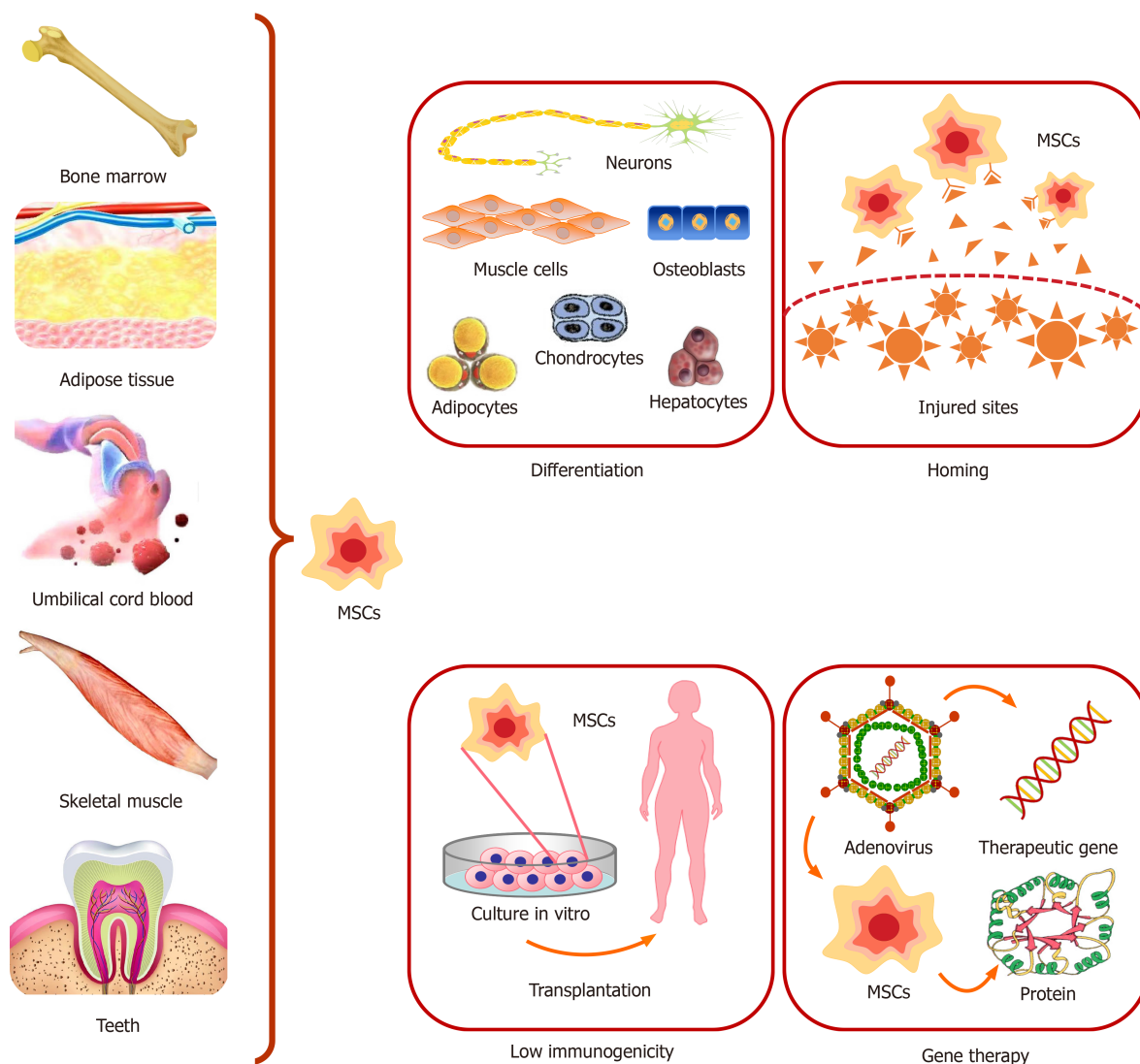


Figure 1 Sources and characteristics of mesenchymal stem cells. Mesenchymal stem cells (MSCs) have a wide range of sources, including adult bone marrow, umbilical cord or placental blood, adipose tissue, skeletal muscle, teeth and other tissues. MSCs have the capacity for multilineage differentiation and self-renewal. They can differentiate into neurons, muscle cells, osteoblasts, chondrocytes, adipocytes, hepatocytes and so on under appropriate conditions. MSCs have the characteristic of homing, the mechanism of which is widely believed to be that sites of injury release various factors, and there are receptors for these factors on the surface of MSCs. MSCs have low immunogenicity and can be cultured and isolated artificially *in vitro* for transplantation. MSCs can also be used as carriers for gene therapy. MSCs can be transfected with therapeutic genes and express the protein of exogenous genes well.

OESOPHAGEAL ULCERATION

Oesophageal ulcers are mostly caused by gastrointestinal reflux disease^[18]. The incidence of gastroesophageal reflux disease has increased significantly in the past decade^[19]. An ulcer is a defect in the mucosa that can deeply penetrate or even perforate the muscle layer. It is generally caused by ischaemia, oxygen radical formation or nutritional transport blockage. Ulcer tissue accumulates a variety of cytokines released by immune cells that play a complex role in various stages of ulcer recovery, including cell proliferation, re-epithelialization, neovascularization and scar formation. The natural recovery of ulcers consists of the development of surrounding cells towards the centre of the ulcer site and reconstruction of the mucosa. However, adult stem cells derived from bone marrow have the ability to differentiate into mature epithelium, which can fill the ulcer area with epithelium and accelerate the process of re-epithelialization^[20,21].

Okamoto *et al*^[22] transplanted bone marrow cells from male donors into four female patients who needed transplantation treatment due to haematological diseases. Epithelial tissue biopsy samples were collected from the recipient's digestive tract through gastroenteroscopy. With immunohistochemistry and fluorescence *in situ* hybridization, it could be determined that there were donor-derived cells in the recipient's epithelial tissue, most of which were epithelial cells and not inflammatory

Table 1 Related stem cell transplantation experiments

Stem cells sources	Transplant recipients	Transplant methods	Results	Ref.
Bone marrow cells from male human donors	Four female human recipients	Bone marrow transplantation	Regeneration of gastrointestinal epithelia	[22]
High K19-expressing MSCs from bone marrow of mice	Mice, <i>H. felis</i> infected mice	Gastric wall injection, blastocyst injection	Contributed to gastric epithelial regeneration and repair.	[23]
MSCs from bone marrow of male rats	Female rats with gastric ulcers	Gastric wall injection surrounding the ulcer	Acceleration of gastric ulcer healing	[24]
MSC sheets from inguinal fat tissue of rabbits	Rabbits with oral mucosal ulcers	MSC sheets transplanted onto mucosal ulceration	Full-thickness mucosal healing and complete basal cell coverage	[26]
Bone marrow cells from mice	Mice with radiation-induced oesophageal injury	Intravenous injection	Repopulation of the irradiated oesophageal squamous epithelium	[32]
DPSCs from rats	Rats with acute radiation-induced oesophageal injury	Intravenous injection (tail vein)	Healing of tissue damage and improvement the oesophageal function	[34]
MSCs from bone marrow of rats	Rats with oesophageal lye burn	Intravenous injection (tail vein)	Differentiation to epithelial and muscle cells	[37]
Double-layered ADSC sheets from the abdominal subcutaneous fat of pigs	Pigs treated with hemi-circumferential ESD	ADSC sheets placed on the wound site with endoscope	Reduced degree of oesophageal stricture and fibrosis development	[47]
CM of AMSCs from the foetal membrane of pregnant women	Pig treated with semi-circumferential ESD	CM gel applied on the wound site with endoscope	Reduced oesophageal fibrosis and inflammation	[48]
MSCs from bone marrow of pigs	Pigs for circumferential replacement of oesophagus	MSC-seeded matrix for circumferential Replacement of oesophagus	Acceleration of epithelial and muscle cell regeneration	[56]
MSCs from bone marrow of rats	Rats for circumferential replacement of oesophagus	MSC-seeded decellularized oesophagus for orthotopic replacement	Regeneration of functional epithelium, muscle fibres, nerves and vasculature	[57]
MSCs from adipose tissue of pigs	Pigs for full thickness circumferential resection of oesophagus	MSC-seeded synthetic grafts implanted into oesophagus	Regrowth of mucosa, submucosa, and smooth muscle layers and blood vessels	[59]
MSCs from bone marrow of human donors	Rats for interposition procedure between the oesophagus and stomach	MSCs and other cells constructing multicellular artificial oesophagus with bio-3D printing transplanted into oesophagus and stomach	Full coverage of inner luminal surface by epithelial cells	[60]
MSCs from bone marrow of human donors, then MSCs transduced with <i>IFN-β</i> gene	Rats inoculated with melanoma cells and MSCs	Intravenous injection (tail vein)	Proliferation of MSCs in tumours and inhibition of malignant cell growth	[66]
MSCs transduced with <i>IFN-α</i> gene	Mice inoculated with melanoma cells	Intramuscular injection	Decrease in tumour cell proliferation and induction of tumour cell apoptosis	[67]
MSCs from bone marrow of human donors, then MSCs transduced with <i>IFN-λ</i> gene	Mice injected with H460 cancer cells	Subcutaneous injection	Induction of tissue necrosis and inhibition of tumour cell growth in lung metastases	[70]
MSCs from bone marrow of human donors, then MSCs modified with <i>TRAIL</i> gene	Mice injected with Eca-109 cancer cells	Directly injection into tumour	Induction of Eca-109 oesophageal cancer cell apoptosis	[72]
MSCs from bone marrow of rats, then MSCs transfected with <i>TRAIL</i> gene	Mice injected with B16F10 cancer cells	Intravenous injection (tail vein)	Reduction of lung metastases and induction of tumour cell apoptosis	[62]
MSCs from adipose tissue of human donors, then MSCs transfected with <i>TRAIL</i> gene	Mice injected with HeLa cells	Flank injection, tumour injection	Inhibition of tumour cell growth	[73]

ADSC: Adipose-derived stromal cell; AMSCs: Amniotic mesenchymal stem cells; CM: Conditional medium; DPSCs: Dental pulp stem cells; ESD: Oesophagic submucosal division; *H. felis*: *Helicobacter felis*; IFN: Interferon; MSCs: Mesenchymal stem cells; TRAIL: Tumour necrosis factor related apoptosis-inducing ligand.

cells[22]. Okumura *et al*[23] demonstrated that MSCs in bone marrow can play a role in mucosal re-epithelialization and repair.

Cytokeratins are a class of proteins that maintain the cellular structure of epithelial

cells. Keratin 19 (K19) is a cytokeratin and can be considered a marker of epithelial cells. Therefore, K19 can be used to identify the transformation of MSCs into epithelial cells. Okumura *et al*^[23] isolated and cultured MSCs with high K19 expression and injected them directly into the gastric wall of mice. After 24 h, MSCs were found to have filled the gastric mucosa. After 4 wk, the expression of specific markers of epithelial cells was found at the site of MSC injection^[23]. Hayashi *et al*^[24] injected rat MSCs into the stomach wall of rats, observed the recovery of gastric ulcers induced by acetic acid and detected the expression of vascular endothelial growth factor (VEGF) produced by the transplanted MSCs in the rats. VEGF is a kind of angiogenesis regulatory factor that can induce the formation of new blood vessels in granulation tissue and is of great significance for ulcer healing^[25]. The application of VEGF antibody can inhibit the therapeutic effect of MSCs on ulcers in a dose-dependent manner^[24]. Therefore, VEGF plays a significant role in promoting angiogenesis in the treatment of ulcers with MSCs. The mechanisms of gastric ulceration and oesophageal ulceration are similar, so the above experimental principle is also applicable to the oesophagus.

Compared with bone marrow-derived stem cells, adipose-derived stem cells (ASCs) are easier and less invasive to obtain and easier to isolate and culture. One study demonstrated that the use of ASC sheets accelerated the healing of oral mucosal ulcers^[26]. In many ischaemic models, ASCs have been shown to increase the capillary density and decrease the inflammatory response^[27]. ASCs can secrete paracrine factors that promote tissue healing^[28], and their differentiation potential is also conducive to the treatment of damaged tissues^[29].

ACUTE RADIATION-INDUCED OESOPHAGEAL INJURY

Radiation therapy for various cancers in the chest, especially lung cancer, will inevitably lead to radiation-induced oesophageal injury^[30]. However, the occurrence of radiation-induced oesophagitis will hinder the treatment of cancer, and there are very few drugs that can protect the oesophagus from radiation^[31]. Therefore, stem cell therapy constitutes an innovative and effective treatment strategy.

Epperly *et al*^[32] simulated a model of radiation-induced oesophageal injury with 30 Gy of radiation. After bone marrow cells were injected intravenously into the model mice, the cells migrated to the oesophageal lesions, differentiated into oesophageal squamous epithelial cells and improved the overall survival rate of the mice. In addition, dental pulp stem cells (DPSCs) are a type of MSC that are used to repair periodontal tissues. In fact, in addition to forming odontoblasts, they can also differentiate into adipose, bone, cartilage, muscle, vascular endothelial, liver and nerve cells, among others, through induction with different cytokines. The isolation and collection of DPSCs is highly non-invasive and inexpensive^[33]. Zhang *et al*^[34] placed iodine 125 (¹²⁵I) particles into a disposable ureteral lumen and introduced them into the oesophagus to create an acute radiation-induced oesophageal injury model. DPSCs cultured *in vitro* were injected into the experimental Sprague Dawley rats through the tail vein. Finally, it was demonstrated that DPSC transplantation was helpful for the treatment of acute radiation-induced oesophageal injury. DPSCs expanded *in vitro* can home to oesophageal lesions to proliferate and trans-differentiate into oesophageal stem cells^[34].

CORROSIVE OESOPHAGEAL INJURY

Corrosive oesophageal injury occurs rarely in adults as an accident, but it is very common among children due to the characteristics of the children themselves, especially in many developing countries, for which there are many social causes^[35]. The mucosal layer of the oesophagus is destroyed when it is exposed to corrosive substances. As the disease progresses, the damage invades the muscular layer. In severe cases, perforation occurs. Self-repair may eventually lead to fibrosis, stenosis and shortening of the oesophagus^[36]. Drugs can be effective to varying degrees, but they cannot lead to much improvement in cases of serious injury.

To test the application of stem cells, Kantarcioglu *et al*^[37] made a standard model of oesophageal caustic injury with lye in 65 Wistar rats. Bone marrow MSCs were obtained from the tibia and femur of rats and cultured *in vitro*. They were injected into the experimental rats through the tail vein. Finally, a histopathological evaluation was performed, including determination of submucosal collagen, mucosal muscle injury, intrinsic muscle injury and collagen deposition as well as calculation of the oesophageal stenosis index. Positron-emission tomography was used to observe the

homing behaviour of the stem cells. The results showed that the structure of the oesophagus was not completely restored, but the stem cells indeed showed homing and differentiation behaviour. The researchers speculated that the less than ideal treatment may be related to the number and location of stem cell injections^[37]. Bone marrow MSCs have the ability to home to damaged tissue and differentiate into various cell types. Pittenger *et al*^[13] and Okamoto *et al*^[22] proved that MSCs can differentiate into epithelial cells in the damaged gastrointestinal tract to promote repair. Oswald *et al*^[38] demonstrated experimentally that bone marrow MSCs can differentiate into endothelial cells *in vitro*, greatly promoting angiogenesis and therefore facilitating tissue repair.

OESOPHAGEAL STRICTURE FORMATION AFTER ENDOSCOPIC SUBMUCOSAL DISSECTION

With the increasing number of patients with oesophageal cancer, endoscopic submucosal dissection (ESD) has become a mature treatment strategy, especially for early oesophageal cancer^[39,40]. However, ESD has many side effects, and the incidence of stricture formation due to the peeled mucosa is very high, especially when more than three-quarters of the oesophagus is stripped. Prophylactic endoscopic balloon dilatation and steroid hormones have traditionally been effective strategies for preventing oesophageal stricture formation, although these methods can cause discomfort and complications^[41]. Regenerative medicine has been widely recognized as a method of treating diseases using the body's own components^[42]. Due to the development of regenerative medicine, techniques using autologous epidermal cell sheet transplantation have been developed to treat stenosis^[43]. This technology preserves the adhesion molecules between cells so that the cells can remain linked together during transplantation and attach to damaged tissue^[44]. For example, cell sheets composed of oral mucosal cells can promote oesophageal epithelial regeneration after ESD^[45].

Adipose-derived stromal cells (ADSCs) are a type of adult stem cell that can differentiate into different types of cells and exert paracrine and angiogenic effects to facilitate tissue repair^[46]. In addition, ADSCs are easy to isolate. Perrod *et al*^[47] performed ADSC sheet transplantation into the oesophagus post-operatively. Compared with the control, ADSC transplantation reduced the incidence of severe stenosis 3 d post-operatively. In addition to stem cells themselves, various secretory factors produced during stem cell differentiation may also have positive effects. Mizushima *et al*^[48] speculated that the therapeutic effect of foetal membrane or amniotic MSC (AMSC) transplantation on various diseases may be attributed to the factors secreted by the AMSCs; thus, conditioned medium (CM) obtained from AMSCs was used to explore its therapeutic effect on post-ESD stenosis. The researchers isolated and cultured AMSCs from the foetal membrane during caesarean section in pregnant women who had provided consent and used them to prepare CM gel. The experiment utilized different gradients and frequencies of CM gel usage to compare the use of steroid drugs. The degree of oesophageal stenosis was assessed by calculating the lateral mucosal constriction rate and performing histological and immunohistochemical examinations. The results demonstrated that CM could reduce fibrosis and inflammation in the oesophagus after surgery^[48].

OESOPHAGEAL RECONSTRUCTION

Oesophageal replacement or resection is required in many diseases, such as long-gap oesophageal atresia, a congenital disease in children, and oesophageal cancer in adults that requires oesophagectomy. Traditionally, oesophageal tissue is often replaced by tissue from the stomach, jejunum and colon. Many post-operative complications, such as stenosis, reflux, delayed emptying, anastomotic fistula and dysfunction, are inevitable and reduce the quality of life to a large extent^[49-51]. On the basis of regenerative medicine technologies, the rise of tissue engineering has greatly improved the treatment of this kind of disease. Tissue engineering integrates engineering and life science and uses scaffolds or a combination of scaffolds and cells to reconstruct the structure or function of tissues or organs^[52]. Scaffolds can be acellular or seeded with cells. However, the transplantation of acellular scaffolds requires advanced materials, which are needed to support the regeneration of corresponding tissues, such as epithelium and muscle. In oesophageal applications, it is possible to seed epithelial cells on the scaffold in advance, which is helpful for epithelialization of the oesophagus, but the muscular layer is difficult to form^[53].

Although many experiments have successfully transplanted autologous smooth muscle cells into the oesophagus, their proliferation capacity is limited^[54].

Zani *et al*^[55] have suggested that stem cells can help with oesophageal regeneration. In a study by Catry *et al*^[56], MSCs promoted the therapeutic effect. They isolated MSCs from the posterior iliac crest by aspiration and cultured them *in vitro*; then, they compared the effect of a stem cell-seeded matrix with that of a non-stem cell-seeded matrix in full-layer oesophageal replacement. The results showed more epithelial cells in the early stage in the transplanted area of the oesophagus along with muscle cell regeneration in the experimental group with stem cells than in the control group without stem cells. Therefore, under the effect of stem cells, the process of epithelial and muscle cell regeneration will be accelerated^[56]. Sjöqvist *et al*^[57] successfully integrated bone marrow MSCs into acellular scaffolds to replace the oesophagus and proved that MSCs can differentiate into oesophageal-related cells. There has now been a clinical case of using tissue engineering to treat an oesophageal defect. The patient underwent commercial stent placement but did not recover as expected. The researchers used extracellular matrix and autologous plasma to help repair the oesophagus. The extracellular matrix can provide an environment for stem cell differentiation and attract and induce stem cells to promote organogenesis. When the stent was removed, endoscopic ultrasonography showed that the newly formed oesophageal wall contained the five normal structural layers. Furthermore, the new oesophagus also achieved a certain degree of functional recovery^[58].

In addition to MSCs, ASCs are easy to obtain and abundant in number and can play an important role in tissue engineering of the oesophagus. According to experiments in which ASCs successfully differentiated into smooth muscle cells, Wang *et al*^[54] implanted ASCs into the muscle layer of an acellular matrix, and the results showed that the ASCs attached to the muscle layer and achieved migration and proliferation. La Francesca *et al*^[59] placed scaffolds loaded with adipose-derived MSCs into a pig model of oesophagectomy, along with a physical stent supporting the oesophageal structure. Histological examination of the oesophagus and an evaluation of oesophageal stenosis were performed. After removal of the physical stent and scaffold, the formation of oesophageal mucosa and muscularis was observed, as was vascularization, and there was no oesophageal stricture formation^[59]. Currently, on the basis of tissue engineering technology, it is possible to use scaffold-free structures composed of a variety of cells to replace the oesophagus using biological 3D printing technology. In cell mixtures, the more MSCs, the better the structure and function of the oesophagus^[60].

GENE THERAPY FOR OESOPHAGEAL CANCER

Oesophageal cancer ranks fifth among the most common cancers in China. Worldwide, 60% of new cases of oesophageal cancer occur in China^[61]. Based on traditional treatment with surgery, radiotherapy and chemotherapy, gene therapy, which has been successfully applied in the treatment of many diseases, has been developed for oesophageal cancer. The function of the therapeutic gene depends on the efficacy of the delivery carrier, and effective delivery to the tumour site is key in gene therapy. Viral vectors were more commonly used in the past, but there were some side effects that could lead to systemic damage, and many new vectors have limited therapeutic effects due to their respective characteristics^[62]. MSCs can still maintain their original characteristics after being successfully transfected with exogenous genes and cultured *in vitro*. MSCs can successfully express exogenous gene products *in vitro* and secrete therapeutic proteins as carriers *in vivo*^[63]. MSCs have been proven to inhibit tumour cell proliferation and tumour angiogenesis by secreting soluble factors *in vitro*^[64]. There are higher levels of paracrine growth factors in tumour tissues than in normal tissues. The proliferation of MSCs requires the presence of these factors^[63]. Hu *et al*^[62] found that stromal cell-derived factor 1 may attract MSCs to migrate to the tumour microenvironment *in vitro*. Therefore, MSCs, being able to migrate to tumour tissues, are also considered to be good carriers for therapeutic genes and anti-tumour biological agents^[65].

Studený *et al*^[66] investigated the effect of MSCs transfected with the interferon β (IFN- β) gene on tumour tissue. Clinical trials have shown that the use of IFN is limited by the systemic maximum tolerable dose and cannot fully exert its anti-tumour effect. However, MSCs transfected with the IFN- β gene can preferentially localize to tumour tissues so that IFN- β is locally released and minimally affects other areas^[66]. MSCs with the IFN- α gene can regulate the activity of immune cells by secreting IFN- α in tumour tissue^[67]. IFN- λ can inhibit cancer cell proliferation by blocking the G1 phase of oesophageal cancer cells^[68]. Li *et al*^[69] found that adenovirus

carrying the IFN- λ gene can induce mitochondrial-mediated oesophageal cancer cell apoptosis in mice. Therefore, it is a feasible method to introduce the IFN- λ gene into stem cells to treat oesophageal cancer. Yang *et al.*^[70] verified that IFN- λ -modified MSCs inhibit the growth of tumour cells by activating the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) molecular pathway. TRAIL is a molecule that can induce the apoptosis of tumour cells, but its short half-life in plasma limits its wide application^[71]. Li *et al.*^[72] introduced the TRAIL gene into human bone marrow-derived MSCs by adenovirus vector and delivered the MSCs into mice by subcutaneous injection on the back. The results showed that the MSCs could induce the apoptosis of oesophageal tumour cells and inhibit the growth of tumour tissue through the expression of TRAIL. Non-viral vectors can also be used to transfect MSCs, which would have no viral interference with the treatment and could improve the targeting effect of MSCs^[62]. Human adipose-derived MSCs are also an effective tool for TRAIL gene therapy for cancer, and the material source is more advantageous^[73].

There are many other tumour-suppressing genes that can be introduced into MSCs for cancer treatment, such as IL-12, IL-24 and pigment epithelium-derived factor. However, few genes have been identified for treating oesophageal cancer, and these can be further developed in the future^[74-76]. There is some debate regarding the effect of stem cells on tumours. Some experiments have shown that MSCs can promote tumour growth, which may be due to different experimental designs, the immunosuppressive effect of stem cells themselves or some cytokines secreted by stem cells^[60].

PROSPECTS

There are still some areas that need to be developed and expanded in the treatment of oesophageal diseases. For example, stem cells can be used as gene carriers or drug carriers directly, and drugs can be released in a targeted manner depending on the function of stem cell homing. The combination of nanocarriers and MSCs will allow better control over the position and mode of drug release^[77]. Additionally, MSC-derived extracellular vesicles (EVs) have good prospects in regenerative medicine techniques^[78]. As phospholipid bilayer vesicles, EVs secreted by MSCs contain many substances, such as enzymes, signal molecules and RNA. There have been many experiments showing that the EVs of MSCs can alleviate or treat many diseases by transmitting the effects of MSCs, including inhibiting the inflammatory response, promoting cell proliferation and promoting angiogenesis and anti-apoptosis ability. Future strategies could include increasing the content of active molecules in EVs, targeting them to damaged tissues, evading the clearance system to improve the action time and using EVs for drug delivery to improve the therapeutic efficiency^[79]. However, there is still much to learn about stem cells. In continuing to explore the benefits of stem cells for treating disease, there are still many uncertain factors and possible risks. In a model of oesophagitis and intestinal metaplasia in female experimental rats, bone marrow stem cells from male rats were injected into the tail vein of the experimental rats. The Y chromosome from the male rat cells could be found in the oesophageal squamous epithelium and metaplasia columnar epithelial cells in the female experimental rats, although the possibility of cell fusion could not be ruled out. However, summarizing similar experimental studies, it is undeniable that stem cells can promote the formation of oesophageal epithelialization and oesophageal metaplasia. It is well known that oesophageal metaplasia is an important process in the formation of Barrett's oesophagus, and Barrett's oesophagus is a risk factor for oesophageal cancer^[80]. In addition, genetically modified MSCs can be used to treat tumours. However, it is controversial whether bone marrow MSCs inhibit or promote tumour tissue activity. Experiments have shown that bone marrow MSCs can inhibit tumour cell proliferation and promote tumour cell apoptosis *in vitro* but can promote tumour growth *in vivo*, which may be related to the promotion of tumour angiogenesis^[81].

CONCLUSION

In summary, the treatment of oesophageal diseases by stem cells can be based not only on their own characteristics, such as multilineage differentiation, self-renewal, low immunogenicity and homing capacity, for the recovery and reconstruction of oesophageal structure and function to a certain extent, but also on integration with other biotechnology, the combination of which has greater therapeutic efficacy than either component alone. Although there have been few clinical trials, the prospects of

stem cells in the treatment of oesophageal diseases are very promising. In addition, the function of stem cells has not yet been completely understood. In the process of stem cell development, there are still many unknown and uncertain factors to be explored.

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Basic Study

Cytotoxicity of nonylphenol on spermatogonial stem cells via phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin pathway

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Abstract

BACKGROUND

With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors. Nonylphenol (NP) is a stable degradation product widely used in daily life and production and has been proven to affect male fertility. However, the underlying mechanisms therein are unclear. Thus, it is necessary to study the effect and mechanism of NP on spermatogonial stem cells (SSCs).

AIM

To investigate the cytotoxic effect of NP on SSCs *via* the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway.

METHODS

SSCs were treated with NP at 0, 10, 20 or 30 μmol . MTT assay was performed to evaluate the effect of NP on the proliferation of SSCs. Flow cytometry was conducted to measure SSC apoptosis. The expression of Bad, Bcl-2, cytochrome-c, pro-Caspase 9, SOX-2, OCT-4, Nanog, Nanos3, Stra8, Scp3, GFR α 1, CD90, VASA, Nanos2, KIT, PLZF and PI3K/AKT/mTOR-related proteins was observed by western blot, and the mRNA expression of SOX-2, OCT-4 and Nanog was detected by quantitative reverse transcription polymerase chain reaction.

RESULTS

additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Compared with untreated cells (0 μ mol NP), SSCs treated with NP at all concentrations showed a decrease in cell proliferation and expression of Bcl-2, Nanog, OCT-4, SOX-2, Nanos3, Stra8, Scp3, GFR α 1, CD90, VASA, Nanos2, KIT, and PLZF ($P < 0.05$), whereas the expression of Bad, cytochrome-c, and pro-Caspase 9 increased significantly ($P < 0.05$). We further examined the PI3K/AKT/mTOR pathway and found that the phosphorylation of PI3K, AKT, mTORC1, and S6K was significantly decreased by NP at all concentrations compared to that in untreated SSCs ($P < 0.05$). NP exerted the greatest effect at 30 μ mol among all NP concentrations.

CONCLUSION

NP attenuated the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress. The associated mechanism may be related to the PI3K/AKT/mTOR pathway.

Key words: Spermatogonial stem cells; Nonylphenol; Cytotoxicity; Phosphatidylinositol-3-kinase; Protein kinase B; Mammalian target of rapamycin

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Core tip: With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors. Nonylphenol is a stable degradation product widely used in daily life and production and has been proven to affect male fertility. Our study demonstrated that nonylphenol reduced the proliferation, differentiation and stemness maintenance of spermatogonial stem cells while promoting apoptosis and oxidative stress. The mechanism may be related to the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin pathway, providing a potential method for the treatment of male infertility.

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INTRODUCTION

Spermatogonial stem cells (SSCs) are a class of primitive spermatogonia located at the basement membrane of convoluted seminiferous tubules that not only self-renew and maintain their population but also differentiate into spermatocytes. SSCs are transformed into primary and secondary spermatocytes, which then produce round and long spermatozoa cells, eventually forming sperm. SSCs are considered to be immortal because they can replicate themselves in adults and pass on from generation to generation.

With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors^[1,2]. Environmental endocrine disruptors are exogenous substances that can interfere with the endocrine functions of the body, including those that mediate the synthesis, secretion, transport, binding, biological effects and clearance of hormones. This in turn leads to endocrine disorders and causes abnormal damage to body behavior, reproduction and development. Nonylphenol (NP) is a stable degradation product of alkylphenol that is widely used in plasticizers, pesticide emulsifiers and other industrial applications. Its molecular structure is similar to that of estradiol in the human body and can interfere with endocrine metabolism and damage human health^[3-5]. Studies have shown that NP affects the body's hormonal balance and damages the body's reproductive functions^[6,7]. NP can reduce the testicular weight and sperm production in rats without inducing pathological changes in the testis^[8], but the underlying mechanisms therein are unclear. In this study, we observed the effect of different concentrations of NP on the proliferation, differentiation and apoptosis of isolated SSCs. Further molecular experiments were

performed to verify whether these effects were achieved *via* the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway.

MATERIALS AND METHODS

SSC culture and NP treatment

Ten-day-old (specific pathogen-free, disinfected with 75% ethanol) neonatal mice were provided by the Hubei Province Disease Control Center (No. 211002300042744). After anesthesia, bilateral testicles were removed from the mice and placed in sterile Petri dishes. Testicular tissue was rinsed three times with phosphate-buffered saline containing 5% penicillin-streptomycin (PAB180086, Bioswamp). Primary SSCs were isolated according to the method of Yu *et al*^[9]. The rinsed testicular tissue was peeled off under a microscope, placed in a Petri dish with a small amount of high-glucose Dulbecco's modified Eagle medium (DMEM, SH30022.01B, Hyclone) and cut into 1-mm³ pieces with surgical ophthalmic scissors. The tissue block was resuspended in a solution of 0.125% trypsin and 0.1% collagenase I (1:1) (17100-017, Gibco) and incubated at 37 °C in an incubator containing 5% CO₂ for 30 min. Tissue digestion was terminated by adding an equal volume of DMEM, and the cells were centrifuged at 2500 × g for 5 min. Thereafter, the cells were resuspended in culture solution and cultured in a Petri dish at 37 °C for 1 h. The cells were then seeded into a feeder-free Petri dish and passaged when they reached 85% confluence.

For flow cytometric identification of SSCs, seven Eppendorf tubes were prepared and 100 µL of single cell suspension was added to each tube. Antibodies against glial cell line-derived neurotrophic factor family receptor alpha-1 (GFRα1, 1:200, ab186855, Abcam), CD90 (11-0903-82, 0.125 µg/test, Invitrogen), VASA (PA5-23378r, 1:200, Invitrogen), Nanos2 (PA5-20553r, 1:50, Invitrogen), KIT (17-1171-82, 0.125 µg/test, Invitrogen) and promyelocytic leukemia zinc finger (PLZF, 1 µg/test, 53-9320-82, Invitrogen) were individually added to each tube (with one blank tube). The tubes were incubated at 4 °C for 45 min in the dark. After the addition of 2 µL of fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (PAB160016, Bioswamp) to each tube, they were further incubated at 4 °C for 45 min in the dark. After adding 400 µL of flow cytometry dyeing buffer to each tube, the cells were subjected to flow cytometry (CytoFLEX S, BECKMAN), and the results were analyzed using CYEXPERT software. After SSCs have been successfully isolated and identified, they were treated with NP (N109556, Aladdin) at 0, 10, 20 and 30 µmol for 7 d based on the method of Huang *et al*^[10]. SSCs were treated with mTOR agonists (MHY1485, 10 µmol) and mTOR inhibitors (AZD8055, 10 µmol) according to the references^[11,12] for 24 h. SSCs were respectively divided into four groups: Control (Control group, not subjected to any activator or inhibitor), NP (NP group), OV or IV (mTOR activator group; mTOR inhibitor group), OV/IV + NP (OV/IV + NP group) groups.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The proliferation of SSCs was detected using an MTT assay kit (PAB180013, Bioswamp) according to the manufacturer's instructions. Cells in the logarithmic growth phase were collected and seeded at 5 × 10³ cells/well in a 96-well plate, and 20 µL of MTT solution was added to each well. The cells were incubated overnight at 37 °C in an incubator containing 5% CO₂, and 150 µL of dimethyl sulfoxide (D2650, Sigma) was added to each well. The optical density was measured at 490 nm with a microplate reader (Multiskan FC, Thermo).

Flow cytometric detection of apoptosis

SSCs (1.0 × 10⁵ cells/mL) were treated once with different concentrations of NP (0, 10, 20 and 30 µmol) in sterile tubes and cultured for 7 d, after which 1 mL of pre-cooled phosphate-buffered saline was added. The cells were centrifuged at 1000 × g. Then, 10 µL of Annexin V-FITC and 10 µL of propidium iodide were added. The cell samples were analyzed by flow cytometry (CytoFLEX S, Beckman Coulter), and one-step fluorescence compensation strategy was used to eliminate interference in the FITC channel. After mTOR overexpression and mTOR inhibitor AZD8055 intervention, the effect of NP on apoptosis of SSCs was also examined.

Western blot

The concentration of proteins extracted from cells was measured using a bicinchoninic acid protein assay kit (PAB180007, Bioswamp). Total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20, incubated with specific

primary antibodies overnight at 4 °C and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:20000, PAB160011, Bioswamp) for 2 h at 4 °C. The following primary antibodies were used: Bad (1:1000, PAB0589, Bioswamp), Bcl-2 (1:100, PAB30041, Bioswamp), cytochrome-c (Cyt-c, 1:1000, ab90529, Abcam), pro-caspase 9 (1:500, ab135544, Abcam), Nanos3 (1:1000, ab216694, Abcam), Stra8 (1:1000, ab49602, Abcam), synaptonemal complex protein 3 (Scp3, 1:1000, ab150292, Abcam), GFR α 1 (1:500, PA1-32476, Thermo), CD90 (1:1000, ab92574, Abcam), VASA (1:1000, ab209710, Abcam), Nanos2 (1:1000, ab70000, Abcam), KIT (1:1000, ab32363, Abcam), PLZF (1:1000, ab39354, Abcam), PI3K (1:1000, ab191606, Abcam), p-PI3K (1:1000, ab182651, Abcam), AKT (1:1000, ab227100, Abcam), p-AKT (1:1000, ab133458, Thermo), mTOR complex 1 (mTORC1, 1:1000, ab137341, Abcam), p-mTORC1 (1:2000, ab226957, Abcam), ribosomal protein S6 kinase (S6K, 1:1000, ab186753, Abcam), p-S6K (1:1000, ab59208, Abcam), sex determining region Y-box 2 (SOX-2, 1:1000, PAB30154, Bioswamp), octamer-binding transcription factor 4 (OCT-4, 1:1000, ab18976, Abcam), Nanog (1:1000, PAB33609, Bioswamp) and GAPDH (1:2000, PAB36264, Bioswamp). After three washes with phosphate-buffered saline/Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:20000, PAB160011, Bioswamp). Protein bands were visualized by enhanced chemiluminescence color detection (Tanon-5200, TANON) and analyzed using AlphaEase FC gel image analysis software.

Quantitative reverse transcription polymerase chain reaction

Whole RNA was extracted from cell samples using Trizol reagent according to the manufacturer's procedures, and cDNA was synthesized using a reverse transcriptase kit (639505, TAKARA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a real-time system (CFX-96, Bio-RAD) with the SYBR Green PCR Kit (KM4101, KAPA Biosystems). Each qPCR reaction was performed in duplicate and the results were analyzed using the $2^{-\Delta\Delta C_t}$ method. The primers were designed and configured by Nanjing Kingsy Biotechnology Co., Ltd. and are listed in [Table 1](#).

Statistical analysis

All data are presented as the mean \pm standard deviation. The SPSS 19.0 software was used for statistical analyses and GraphPad Prism 5.0 was used to prepare the figures. The data were evaluated for statistical significance using one-way analysis of variance. Statistical significance was established at $P < 0.05$.

RESULTS

Culture and identification of mouse SSCs

We observed SSCs on the 3rd and 10th d of culture with optical microscopy. As shown in [Figure 1A](#), the isolated SSCs were round with large and round nuclei and less cytoplasm. Compared with the 3rd d, the number of SSCs increased significantly on the 10th d. We further identified SSCs by flow cytometry. As shown in [Figure 1B](#), the SSCs showed high expression of GFR α 1, CD90, VASA, Nanos2, KIT and PLZF, suggesting that mouse SSCs were successfully isolated and cultured.

NP inhibited the proliferation of SSCs

After SSCs were treated with different concentrations of NP (0, 10, 20 and 30 μ mol) for 7 d, we examined the changes in cell proliferation ([Figure 2](#)). Compared with untreated cells (0 μ mol), the proliferation of cells treated with NP at 10, 20 and 30 μ mol NP decreased significantly ($P < 0.05$) in a concentration-dependent manner. This suggests that NP had an inhibitory effect on SSC proliferation.

NP promoted apoptosis in SSCs

To study the effect of NP on SSCs apoptosis, flow cytometry and western blot were performed. As the NP concentration increased, the percentage of SSC apoptosis increased gradually ([Figure 3A](#)). Examination of the expression of apoptosis-related proteins revealed that compared to untreated SSCs (0 μ mol NP), those treated with 10, 20 and 30 μ mol NP showed a significant increase in the expression of Bad, Cyt-c and pro-Caspase 9 ($P < 0.05$), while the expression of Bcl-2 decreased significantly ($P < 0.05$) ([Figure 3B](#)). We next observed the cell apoptosis after mTOR activator or inhibitor intervention, the results showed that the apoptosis rate of NP group decreased significantly after increased mTOR activity, while the apoptosis rate of SSCs in the NP group increased significantly after inhibiting mTOR activity ([Figure 3C](#)). The expression levels of apoptosis-related proteins are similar to the above trend ($P < 0.05$) ([Figure 3D](#)).

Table 1 Primer sequences

Primer	Sequence, 5'-3'
OCT-4-F	CACTCACATCGCCAATC
OCT-4-R	AAGGTGTCCTGTAGCC
SOX-2-F	AGCGGCGTAAGATGGC
SOX-2-R	CGGTCTCGGACAAAAGT
Nanog-F	AGCCCTGATCTCTACC
Nanog-R	TGAAACCTGTCCTGAGT
GAPDH-F	CCACTCCTCCACCTTTG
GAPDH-R	CACCACCCTGTTGCTGT

NP inhibited stemness maintenance and differentiation of SSCs

As shown in [Figure 4](#), the mRNA and protein expression of stemness maintenance markers Nanog, OCT-4 and SOX-2 in SSCs treated with 10, 20 and 30 μmol NP was decreased compared with those in untreated cells ($P < 0.05$), and the decrease was directly proportional to the NP concentration. Further detection of SSC differentiation markers showed that the protein expression of Nanos3, Stra8, Scp3, GFR α 1, CD90, VASA, Nanos2, KIT and PLZF in SSCs treated with NP was decreased significantly ($P < 0.05$) compared with that in untreated cells ([Figure 5](#)). These results indicated that NP inhibited the stemness maintenance and differentiation of SSCs.

Effect of NP on PI3K/AKT/mTOR pathway

To investigate the influence of NP on PI3K/AKT/mTOR signaling in SSCs, we measured the protein expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K using western blot ([Figure 6](#)). We observed that compared with untreated cells (0 μmol), the expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K was significantly decreased by NP at all concentrations ($P < 0.05$), with 30 μmol NP exerting the greatest effect among all concentrations. We further observed the effect of NP on the PI3K/AKT/mTOR pathway in SSCs after activation or inhibition of mTOR. The results showed that after activation of mTOR, the expression of PI3K, AKT and mTORC1 protein in the NP group decreased significantly compared with the control group ($P < 0.05$), while that in the NP + OV group increased significantly ($P < 0.05$). When mTOR was inhibited, the trend reversed ($P < 0.05$) ([Figure 7](#)).

DISCUSSION

Numerous self-renewal systems exist in male mammals, but in terms of species continuity, the most important process is spermatogenesis, which is crucial in gene transmission for the generation and evolution of male gametes. A large number of sperm is produced *via* spermatogenesis through orderly and strict cell proliferation and differentiation, and SSCs form the basis of this system. NP is an important fine chemical raw material and intermediate that is mainly used in the production of surfactants, antioxidants, lubricant additives, pesticide emulsifiers, resins and rubber stabilizers and is known as a “sperm killer”^[13]. NP has certain toxic effects on sperm, which are differentiated from SSCs. Shaliutina *et al*^[14] found that NP induced oxidative stress in fish sperm, reduced sperm activity and destroyed the integrity of the sperm cell membrane. Meng *et al*^[15] found that NP decreased the number and activity of sperm and increased the rate of sperm deformity with significant NP concentration-dependent response. Though the impact of NP on sperm health has been demonstrated, whether NP has similar toxic effects on SSCs is unclear.

While SSCs can maintain their relative number by self-proliferation, they also undergo mitosis and meiosis to form spermatocytes, which eventually form sperm. In addition, they have the potential to maintain self-renewal and proliferation to cope with possible damage^[16]. The lifetime amplification and immortality of SSCs guarantee sperm production. In this experiment, we treated SSCs with different concentrations of NP for 7 consecutive days. We found that with the increase in NP concentration, the proliferation of SSCs decreased while apoptosis increased gradually. We detected apoptosis-related proteins and found that the pro-apoptotic proteins Bad, Cyt-c and pro-Caspase 9 were upregulated significantly, while the anti-apoptotic protein Bcl-2 was downregulated, suggesting that NP promoted apoptosis and inhibited SSC proliferation by inducing oxidative stress.

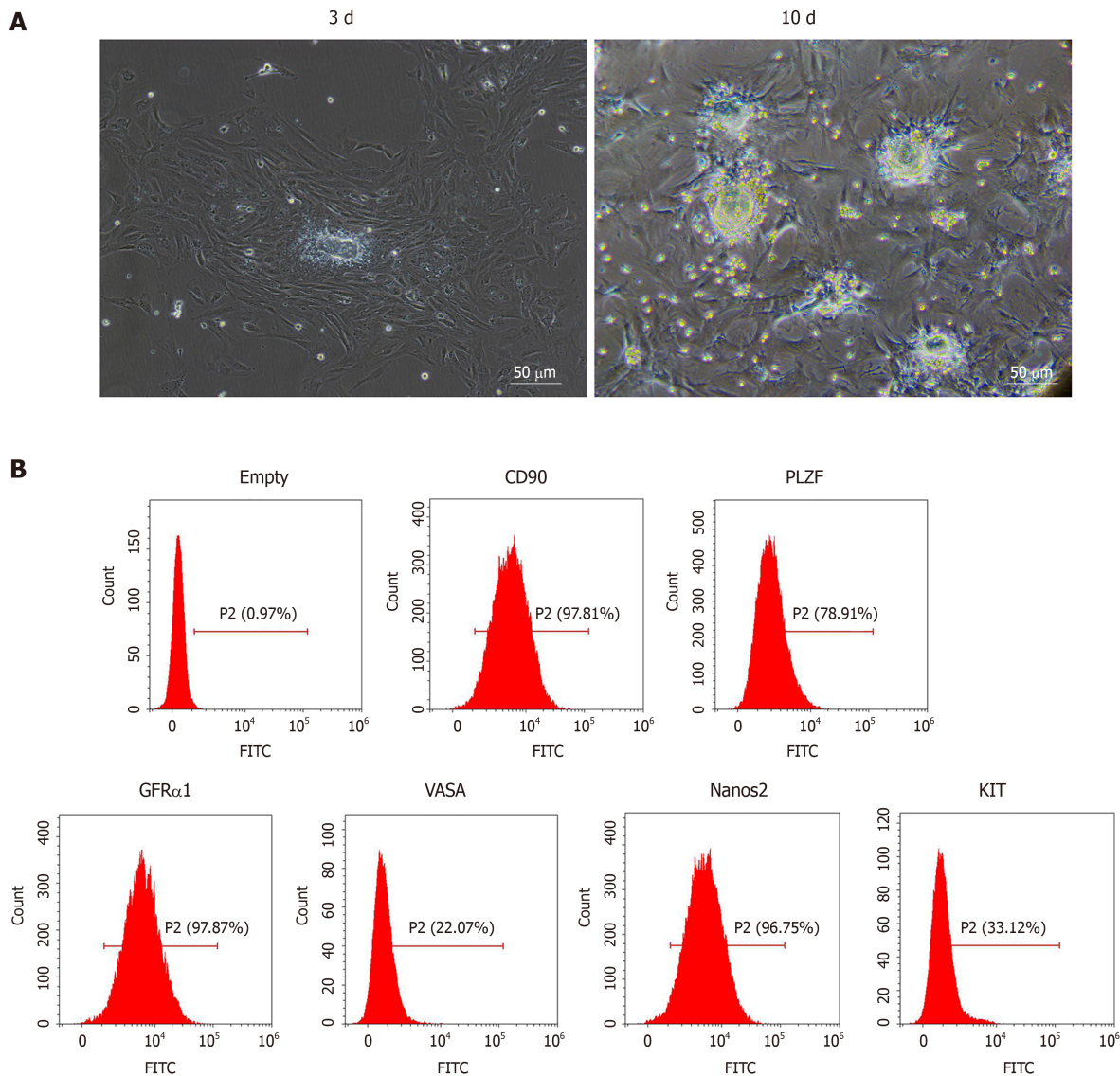


Figure 1 Culture and identification of spermatogonial stem cells. A: Light microscopy of spermatogonial stem cells cultured for 3 d and 10 d (scale bar = 50 μ m); B: Identification of spermatogonial stem cells by flow cytometry.

Stemness maintenance and differentiation potential are important characteristics of SSCs, and the continuous differentiation of SSCs is necessary for spermatogenesis. We showed that NP decreased the expression of markers of SSC stemness maintenance and differentiation. Stra8, which can be specifically activated by retinoic acid, is expressed in mouse embryonic ovary and adult testis, as well as before meiosis in mammalian germ cells^[17,18]. Male Stra8-knockout mice were sterile and had severe defects in spermatogenesis and decreased testicular volume, suggesting that Stra8 plays a vital role in spermatogenesis^[19]. Anderson *et al.*^[20] confirmed that Stra8 is not necessary for DNA replication in pre-filament spermatocytes but is essential for chromosome condensation and DNA homologous recombination in pre-filament spermatocytes during meiosis. Scp3 is an essential component of synaptonemal complex formation that plays an important role in male germ cell meiosis^[21]. Nanos3 is a homologous gene of *Drosophila* Nanos that is specifically expressed in mouse germ cells. It is also an endogenous factor that initiates the process of male germ cell differentiation and plays a critical role in germ cell meiosis^[22,23]. In this study, NP downregulated the expression of Stra8, Nanos3 and Scp3, suggesting that NP may inhibit the differentiation and stemness maintenance of SSCs by regulating pre-meiotic factors.

The PI3K/AKT/mTOR signaling pathway is essential for the proliferation of SSCs. The AKT/mTOR signaling pathway plays an important role in spermatogenesis by regulating the growth and transport of sperm cells in the testis and maintaining the normal function of SSCs^[24,25]. The mTOR signal is directly involved in the late differentiation of SSCs. Inhibition of mTOR expression can lead to the accumulation of

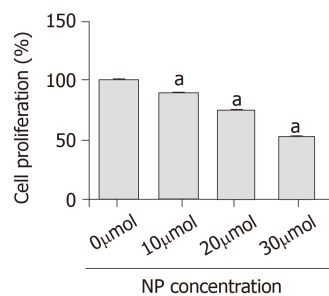
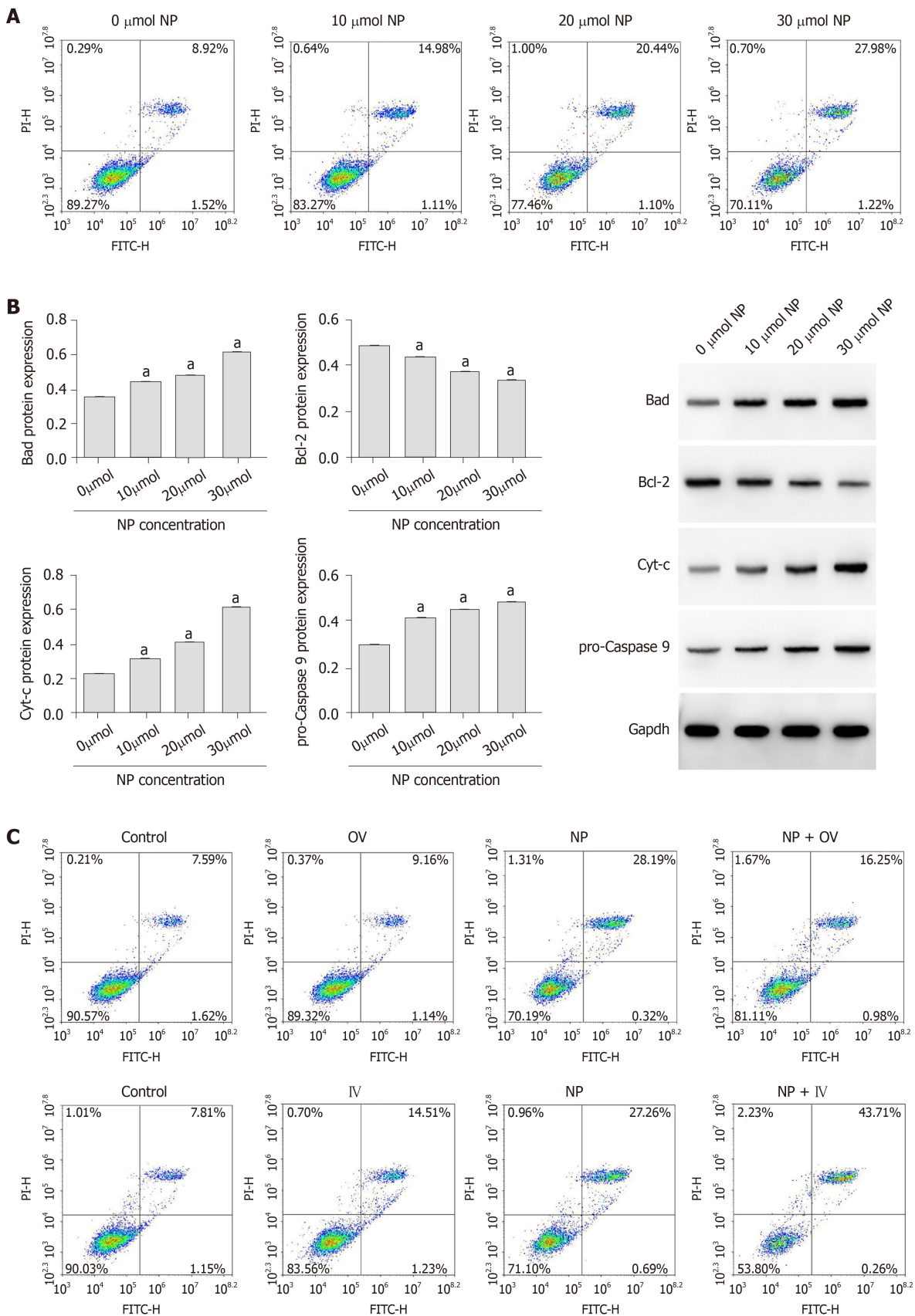


Figure 2 MTT assay of cell proliferation. The results are presented as the mean \pm standard deviation, $n = 3$. ^a $P < 0.05$ vs 0 μmol nonylphenol. NP: Nonylphenol.

a large number of SSCs^[26,27], and the promotion of mTOR expression can promote the proliferation of SSCs^[28,29]. Duan *et al.*^[30] observed that NP inhibits the expression of mTOR and mediates autophagy, apoptosis and necrosis of sperm cells. Duan *et al.*^[31] also revealed that NP inhibits the activity of mTOR-p70S6K/4EBP1 signaling pathway and inhibits sperm differentiation and proliferation. Ma *et al.*^[32] found that Lin28a, a conserved RNA-binding protein, is involved in SSC development, pluripotency, stemness maintenance, proliferation and self-renewal through the PI3K/AKT/mTOR pathway. Huang *et al.*^[33] observed that exposure to NP during pregnancy led to endocrine dysfunction in male offspring, inhibition of AKT/mTOR signaling and induction of apoptosis and autophagy in testicular tissue. We showed that NP significantly reduced the expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K, suggesting that the effect of NP may be exerted through the PI3K/AKT/mTOR signaling pathway.

NP is produced by biodegradation of NP polyoxyethylene ether and is soluble in water. NP polyoxyethylene ether is widely used in industry and household. NP is widely found in rivers and silts in Japan, the United States, Germany and other countries. In heavily polluted rivers in the United Kingdom, the concentration of NP is as high as 534 $\mu\text{g/L}$ ^[34-36]. Similar findings have been found in some rivers in China, and the pollution of NP in China's current water system has significantly exceeded that in foreign countries. Compared with the significant increase in the concentration of NP in water, the reproduction rate of wild animals and humans is greatly reduced^[37,38]. More and more families are childless, and women have been criticized for their inability to reproduce. Gender discrimination has become increasingly serious worldwide. Male oligospermia or azoospermia is also one of the important reasons for the lack of offspring, which is often overlooked. In this experiment, we confirmed that NP can not only reduce the number and activity of sperm but also affect the proliferation and differentiation of SSCs. The mechanism may be related to the PI3K/AKT/mTOR pathway. Therefore, we speculate that promoting the activity of the PI3K/AKT/mTOR pathway may help relieve male oligozoospermia caused by NP.

In conclusion, our study demonstrated that NP reduced the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress, and the mechanism may be related to the PI3K/AKT/mTOR pathway. In future studies, we will investigate whether promoting PI3K/AKT/mTOR activity has therapeutic effects on NP-induced oligospermia.



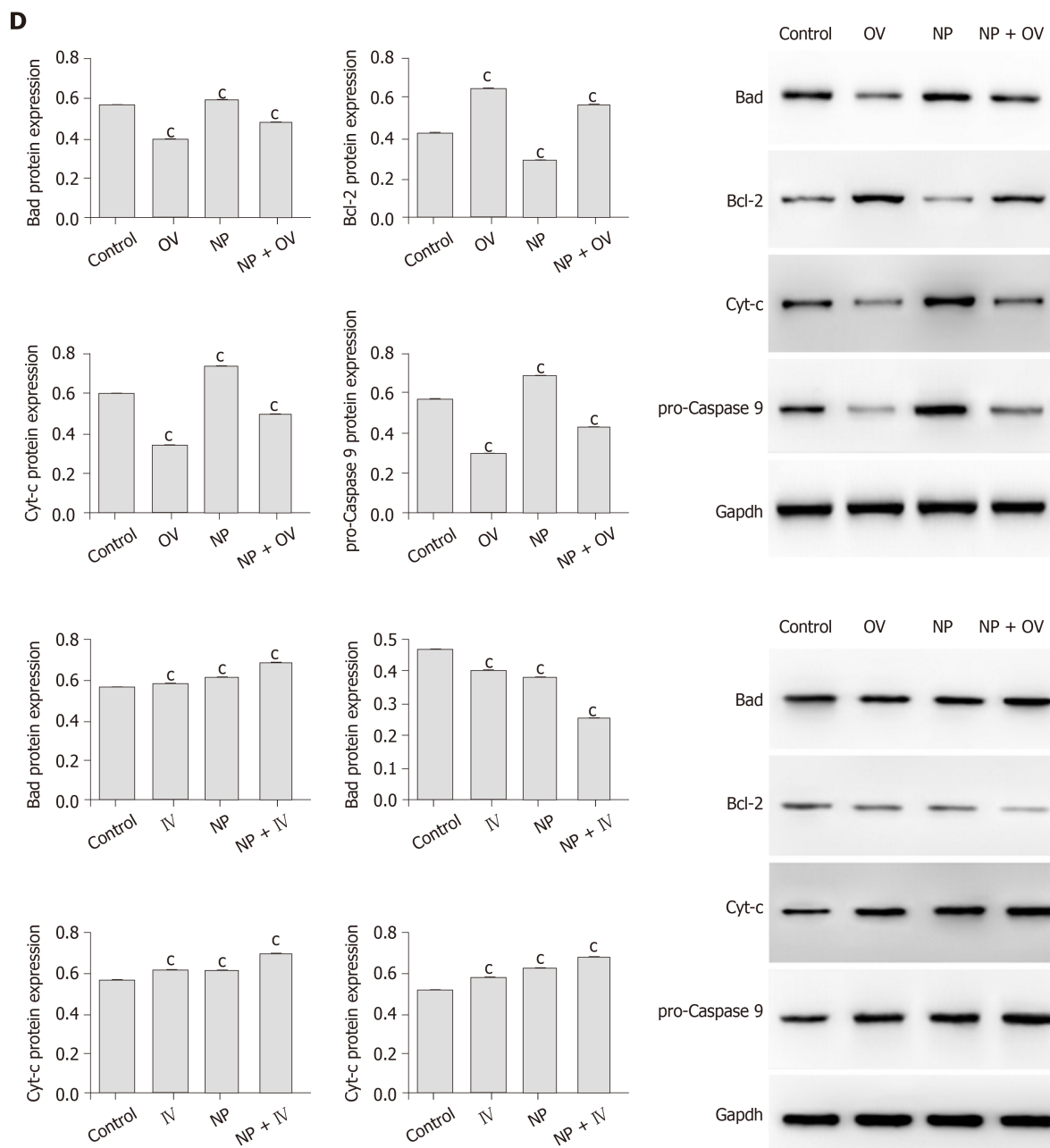


Figure 3 Effect of nonylphenol on apoptosis and oxidative stress in spermatogonial stem cells. A: Detection of apoptosis by flow cytometry; B: Western blot of the protein expression of Bad, Bcl-2, Cyt-c, and pro-Caspase 9. The results are presented as the mean \pm standard deviation, $n = 3$. $^aP < 0.05$ vs $0 \mu\text{mol NP}$; C: Effect of nonylphenol on spermatogonial stem cell apoptosis after mammalian target of rapamycin activation or inhibition; D: Expression of apoptosis-related protein detected by western blot. The results are presented as the mean \pm standard deviation, $n = 3$. $^cP < 0.05$ vs control. NP: Nonylphenol; Control: Control group, not subjected to any activator or inhibitor; NP: Nonylphenol group; OV: Mammalian target of rapamycin activator group; OV + NP: OV + NP group.

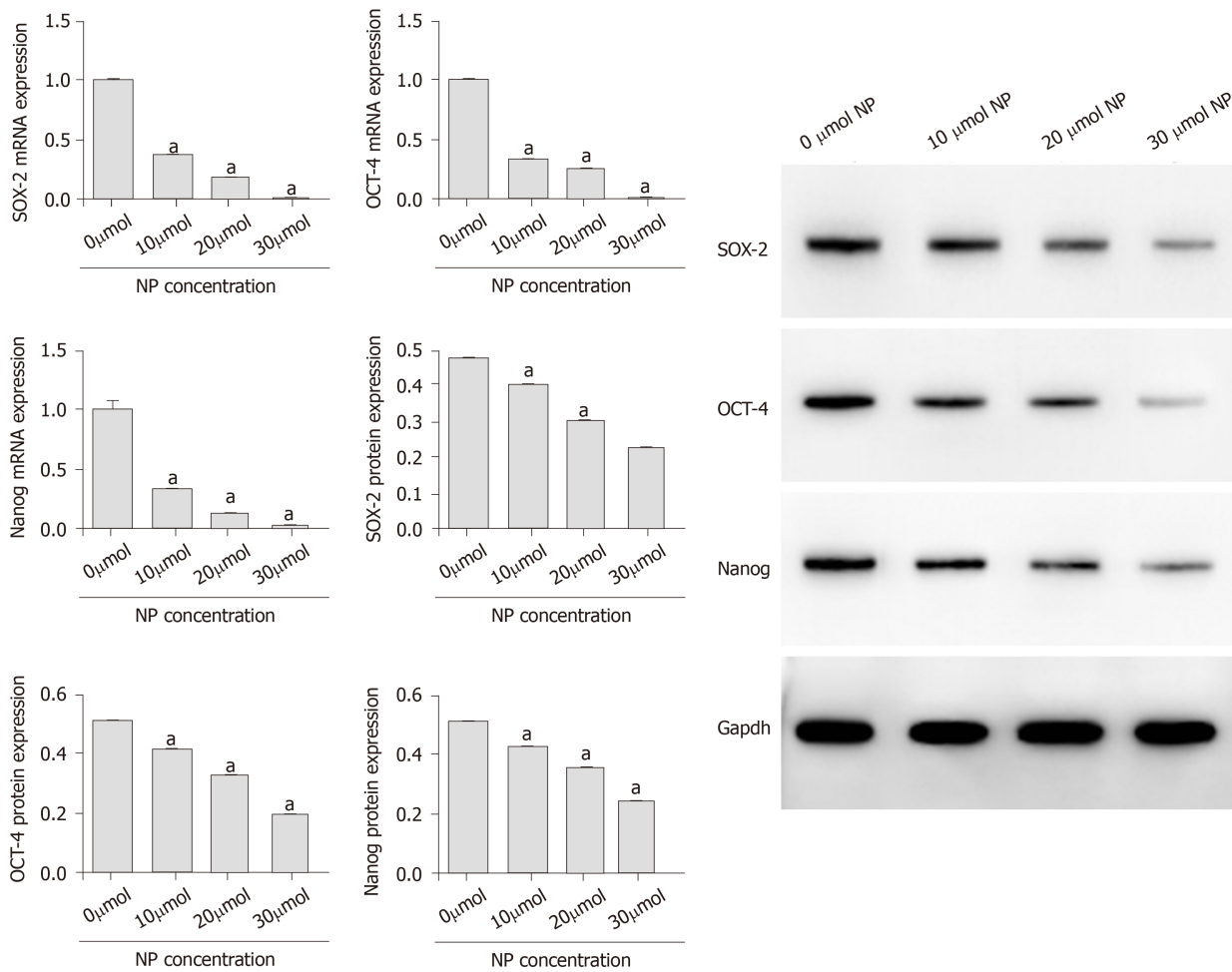


Figure 4 Effect of nonylphenol on stemness maintenance of spermatogonial stem cells. mRNA and protein expression of SOX-2, OCT-4 and Nanog were measured by quantitative reverse transcription polymerase chain reaction and western blot, respectively. The results are presented as the mean \pm standard deviation, $n = 3$. ^a $P < 0.05$ vs 0 μ mol nonylphenol. NP: Nonylphenol.

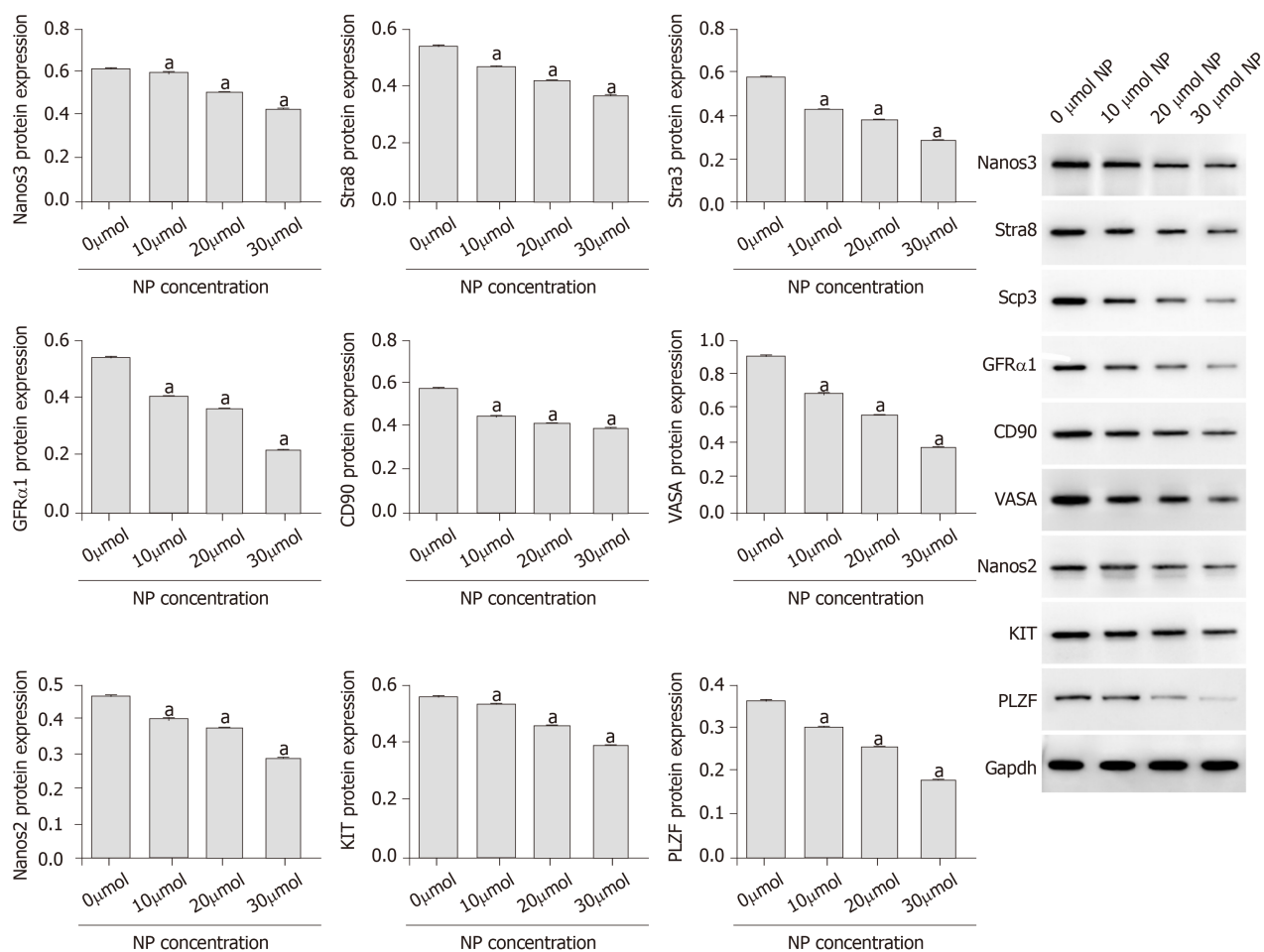


Figure 5 Effect of nonylphenol on differentiation of spermatogonial stem cells. Protein expression of Nanos3, Stra8, Scp3, GFRα1, CD90, VASA, Nanos2, KIT and PLZF was measured by western blot. The results are presented as the mean ± standard deviation, $n = 3$. ^a $P < 0.05$ vs 0 μmol nonylphenol. NP: Nonylphenol.

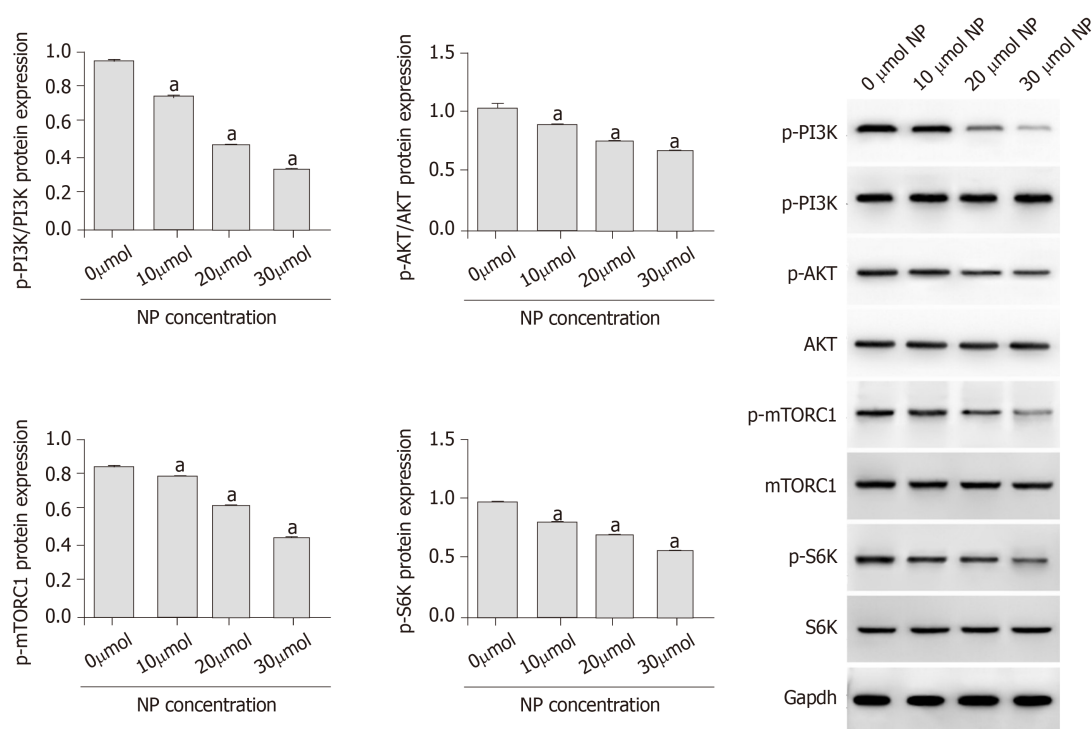


Figure 6 Effect of nonylphenol on the expression of proteins associated with phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin signaling. The results are presented as the mean ± standard deviation, $n = 3$. ^a $P < 0.05$ vs 0 μmol nonylphenol. NP: Nonylphenol.

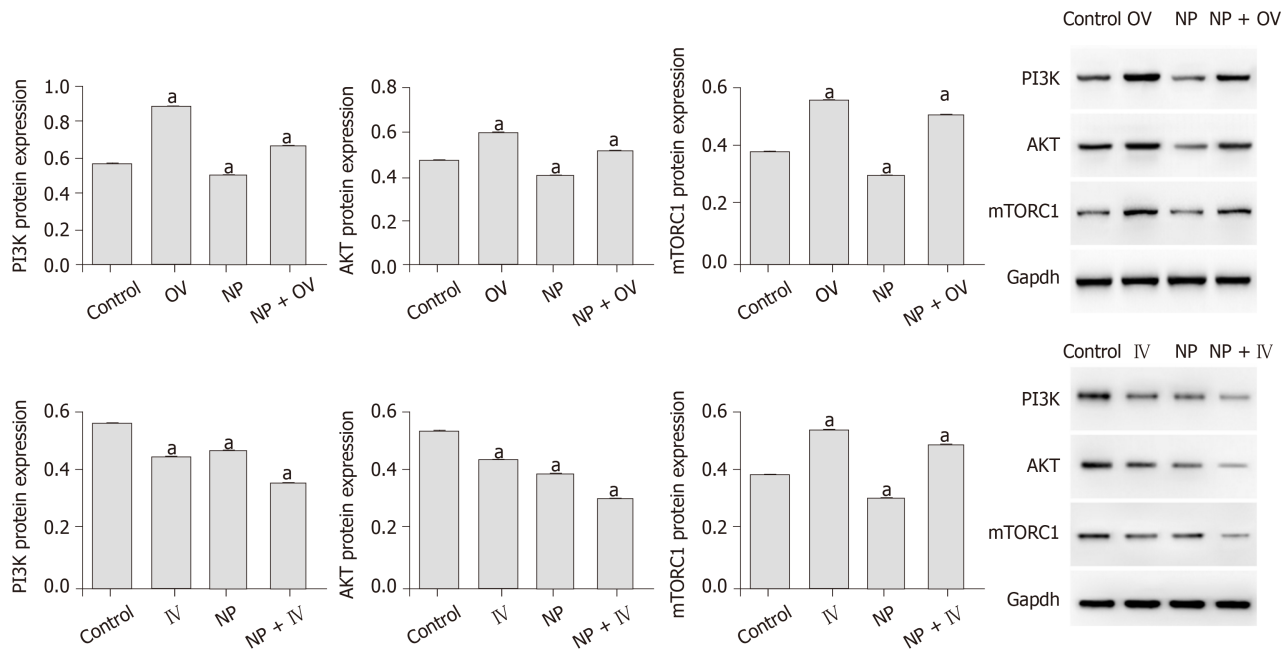


Figure 7 Effects of nonylphenol on phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway after mammalian target of rapamycin activation or inhibition. The results are presented as the mean \pm standard deviation, $n = 3$. ^a $P < 0.05$ vs Control. Control: Control group, not subjected to any activator or inhibitor; NP: Nonylphenol group; OV: Mammalian target of rapamycin activator group; IV: Mammalian target of rapamycin inhibitor group; OV + NP: OV + NP group; IV + NP: IV + NP group.

ARTICLE HIGHLIGHTS

Research background

Infertility has become a social problem that needs to be solved urgently in the world. Apart from the infertility caused by female causes, infertility caused by male oligozoospermia has gradually been valued. Previous studies have confirmed that nonylphenol (NP) widely used in daily life can reduce male sperm counts, but the underlying mechanism is still unclear. Studying the specific mechanism of NP-induced oligospermia could provide some ideas for the treatment of NP-induced oligospermia.

Research motivation

NP has been shown to affect sperm activity, but the mechanism is currently unknown. Spermatogonial stem cells (SSCs) can eventually differentiate into sperm. We aim to study whether NP can affect the proliferation, differentiation and potential mechanism of SSCs in order to provide ideas for clinical treatment of male oligospermia caused by NP.

Research objectives

To study the effect and potential mechanism of NP on SSCs.

Research methods

SSCs were treated with NP at 0, 10, 20 or 30 μmol . MTT was used to detect the effect of NP on the proliferation of SSCs. Flow cytometry, reverse transcription polymerase chain reaction and western blot were used to detect the effect of NP on the proliferation, apoptosis, oxidative stress and stemness maintenance of SSCs. The effects of NP on phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway was also measured by western blot.

Research results

Different concentrations of NP (10, 20 or 30 μmol) could inhibit the proliferation of SSCs, reduce the expression of cell differentiation and stem maintenance related factors and promote apoptosis and the release of oxidative stress factors. We further examined the effect of NP on the PI3K/AKT/mTOR pathway, and the results showed that NP can significantly inhibit the activity of the PI3K/AKT/mTOR pathway. Among all NP concentrations, 30 μmol had the greatest effect.

Research conclusions

NP reduced the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress, and the mechanism may be related to the PI3K/AKT/mTOR pathway, providing a potential method for the treatment of male infertility.

Research perspectives

In this study, we demonstrated *in vitro* that NP could promote apoptosis and oxidative stress of SSCs and reduce the proliferation, differentiation and stem maintenance of SSCs, and the mechanism may be related to the PI3K/AKT/mTOR pathway. Therefore, we speculate that promoting the activity of the PI3K/AKT/mTOR pathway may help relieve male oligozoospermia caused by NP, and we will use PI3K/AKT/mTOR pathway agonist to verify our conjecture in the following studies *in vivo*.

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Retrospective Study

High tibial osteotomy with human umbilical cord blood-derived mesenchymal stem cells implantation for knee cartilage regeneration

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Author contributions: Song JS performed the surgeries; Hong KT performed radiological evaluation; Kim NM, Jung JY, and Park HS analyzed the data; Kim YJ performed statistical analysis; Kong CG and Chang KB contributed to the conception of the study and search of the background literature; Kim SJ designed the study and wrote the manuscript.

Institutional review board

statement: This study was reviewed and approved by the institutional review board of the Korea Ministry of Health and Welfare (2019-3100-003). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Informed consent was obtained

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Abstract

BACKGROUND

High tibial osteotomy (HTO) is a well-established method for the treatment of medial compartment osteoarthritis of the knee with varus deformity. However, HTO alone cannot adequately repair the arthritic joint, necessitating cartilage regeneration therapy. Cartilage regeneration procedures with concomitant HTO are used to improve the clinical outcome in patients with varus deformity.

AIM

To evaluate cartilage regeneration after implantation of allogenic human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) with concomitant HTO.

METHODS

Data for patients who underwent implantation of hUCB-MSCs with concomitant HTO were evaluated. The patients included in this study were over 40 years old, had a varus deformity of more than 5°, and a full-thickness International Cartilage Repair Society (ICRS) grade IV articular cartilage lesion of more than 4 cm² in the medial compartment of the knee. All patients underwent second-look arthroscopy during hardware removal. Cartilage regeneration was evaluated macroscopically using the ICRS grading system in second-look arthroscopy. We also assessed the effects of patient characteristics, such as trochlear lesions, age, and lesion size, using patient medical records.

from all individual participants included in the study.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: All data included in the manuscript are available upon request.

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RESULTS

A total of 125 patients were included in the study, with an average age of 58.3 ± 6.8 years (range: 43-74 years old); 95 (76%) were female and 30 (24%) were male. The average hip-knee-ankle (HKA) angle for measuring varus deformity was $7.6^\circ \pm 2.4^\circ$ (range: 5.0 - 14.2°). In second-look arthroscopy, the status of medial femoral condyle (MFC) cartilage was as follows: 73 (58.4%) patients with ICRS grade I, 37 (29.6%) with ICRS grade II, and 15 (12%) with ICRS grade III. No patients were staged with ICRS grade IV. Additionally, the scores [except International Knee Documentation Committee (IKDC) at 1 year] of the ICRS grade I group improved more significantly than those of the ICRS grade II and III groups.

CONCLUSION

Implantation of hUCB-MSCs with concomitant HTO is an effective treatment for patients with medial compartment osteoarthritis and varus deformity. Regeneration of cartilage improves the clinical outcomes for the patients.

Key words: Allogeneic; Human umbilical cord blood-derived mesenchymal stem cells; Cartilage regeneration; High tibial osteotomy; Osteoarthritic knees; Arthroscopy

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Core tip: This is the first study to evaluate clinical outcomes and cartilage regeneration *via* second-look arthroscopy after implantation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) with concomitant high tibial osteotomy (HTO) for treatment of osteoarthritic knee with varus deformity. HTO treatment of medial compartment osteoarthritis of the knee with varus deformity alone does not sufficiently repair arthritic joints. However, HTO decreases pressure in the medial compartment, providing an environment in which damaged cartilage can be regenerated *via* implantation of allogenic hUCB-MSCs. hUCB-MSC implantation with HTO is an effective treatment for patients with osteoarthritis of the knee with varus deformity, leading to improved clinical outcomes.

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INTRODUCTION

Increased load bearing on the medial compartment of the knee joint in varus deformity, abnormally activates chondrocytes, osteoblasts, and synoviocytes. These cells, then, aberrantly secrete several inflammatory-response proteins and matrix-degrading enzymes, thereby contributing to the gradual progression of medial compartment osteoarthritis (MCOA)^[1-4]. High tibial osteotomy (HTO), a well-established method for treating MCOA, provides an environment in which damaged cartilage can be regenerated *via* decreased medial compartment pressure^[5-7]. HTO alone offers excellent short- and mid-term outcomes, however, these outcomes tend to deteriorate over time^[6-10]. Cartilage regeneration procedures, such as microfracture (MFx) and autologous chondrocyte implantation (ACI) with concomitant HTO, may improve long-term outcomes in this patient population^[11-17]. Although MFx and ACI are widely used for cartilage regeneration, they are not suitable for osteoarthritis (OA) therapy^[18-20].

Injection or implantation of mesenchymal stem cells (MSCs) with concomitant HTO has been reported to regenerate cartilage in MCOA^[21-24]. MSCs can be obtained from the bone marrow (BM), synovium, adipose tissue, and umbilical cord. These cells possess anti-inflammatory, anti-apoptotic, and anti-fibrotic properties. Moreover, MSCs facilitate chondrogenesis, demonstrate a remarkable safety profile without tumorigenicity, and have been shown to improve clinical outcomes in patients with OA^[25-31]. Among the variously sourced MSCs, human umbilical cord blood-derived MSCs (hUCB-MSCs) have shown superior cartilage repair without bone formation or

degeneration of the repaired cartilage^[32-34]. hUCB-MSCs are additionally advantageous because of their high expansion capacity, non-invasive harvesting, and hypo-immunogenicity. Moreover, as hUCB-MSCs are an allogeneic cell source, they are produced as an off-the-shelf product, and can supply sufficiently high numbers of pure stem cells with respect to the cartilage defect area being treated^[35-37]. However, reports on the clinical application of hUCB-MSCs are scarce, and no studies have examined the use of hUCB-MSCs with concomitant HTO^[38,39].

To demonstrate whether regenerated cartilage affects clinical outcomes, herein we retrospectively evaluated clinical outcomes and cartilage regeneration *via* second-look arthroscopy following implantation of hUCB-MSCs with concomitant HTO. In addition, we investigated whether patient characteristics, such as articular cartilage lesions on the patellofemoral joint, age, and cartilage defect size, influence clinical outcomes.

MATERIALS AND METHODS

Participants and study design

We retrospectively reviewed the medical records of patients who underwent second-look arthroscopy during hardware removal after receiving implantation of hUCB-MSCs with concomitant HTO for the treatment of MCOA between January 2014 and November 2016. The study protocol was approved by the institutional review board of Korea Ministry of Health and Welfare (2019-3100-003). The patients included in this study were over 40 years old, and had a varus deformity of more than 5° and a full-thickness International Cartilage Repair Society (ICRS) grade IV articular cartilage lesion of more than 4 cm² in the medial compartment of the knee^[40] (Figures 1 and 2A). Patients with grade IV OA of the medial compartment (identified by radiological assessment according to Kellgren and Lawrence system^[41]), knee ligament injuries, metabolic arthritis, joint infections, and articular cartilage lesions at the lateral compartment were excluded. Herein, we evaluated clinical outcomes 3 years post-surgery and assessed cartilage regeneration *via* second-look arthroscopy. Effects of regenerated cartilage on clinical outcomes were evaluated after classification according to the ICRS grading system. We also assessed the effects of patient characteristics such as patient age, presence of a trochlear lesion, and lesion size of medial femoral condyle (MFC) from the patient's medical records.

Preparation of hUCB-MSCs

CARTISTEM® (Medipost, Seongnam-si, Gyeonggi-do, South Korea), an off-the-shelf medicinal product for cartilage regeneration, was used in the study. This product, which consists of 1.5 mL hUCB-MSCs (7.5×10^6 cells/vial) and 4% hyaluronic acid (HA) hydrogel, was approved for cartilage regeneration by the Korea Food and Drug Administration in January 2012. The therapeutic dose is 500 μ L/cm² as specified in the manufacturer's instructions. Preoperatively, the cartilage defect size was measured by magnetic resonance imaging (MRI), and the therapeutic dose was determined. After combining hUCB-MSCs with 4% HA hydrogel using a spatula, the mixture was transferred into a 5-mL syringe for implantation into the defect.

Surgical procedure and postoperative management

All surgical procedures, including diagnostic arthroscopy, synovectomy, excision of degenerative menisci tears, microfracture, and HTO, were performed by a single surgeon. After completion of the arthroscopic procedure, the fluid was washed out, and arthroscopic instruments were removed from the joint. A 5-7 cm longitudinal incision was made on the medial parapatellar area, and the medial femoral condyle (MFC) was exposed by dissecting the medial patellofemoral ligament and joint capsule. The damaged cartilage was removed using a curette, and sclerotic bone on the surface of the femoral condyle was removed using a burr. For implantation of hUCB-MSCs, multiple holes (4 mm in diameter and 4 mm in depth) were made in cartilage defects, and the space between the holes was drilled using a 2-mm-thick Kirschner wire. Irrigation was used to remove intra-articular debris. The hUCB-MSC and HA hydrogel mixture was then implanted into the holes and articular surface (Figure 3A-C). After implantation of hUCB-MSC, open-wedge HTO was performed using an anatomical locking metal-block plate (Ohtofix; Ohtomedical CO. Ltd., Goyang-si, South Korea)^[42]. All knees underwent uniplanar osteotomy aiming to correct the mechanical axis to approximately 62° lateral to the tibial plateau^[43] (Figure 2B). After surgery, patients were encouraged to perform isometric quadriceps/hamstring exercise and straight leg-raises, however, knee flexion was limited to 90° for 4 wk. Partial weight-bearing began after 4 wk, and full weight-

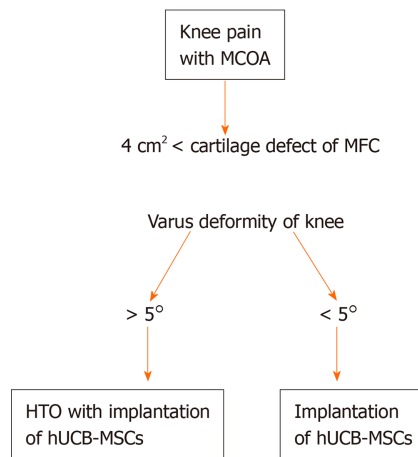


Figure 1 Flow chart of selection criteria. MCOA: Medial compartment osteoarthritis; MFC: Medial femoral condyle; HTO: High tibial osteotomy; hUCB-MSCs: Human umbilical cord blood-derived mesenchymal stem cells.

bearing was permitted at week 6.

Clinical outcome assessment

The clinical outcomes of all patients were evaluated preoperatively, as well as at 1 year, 2 years, and 3 years postoperatively. The guidelines of the International Knee Documentation Committee (IKDC) were used to evaluate knee function and sport activity^[44], while the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score was used to evaluate OA^[45]. Scores obtained using visual analog scales (VAS) were also used to assess pain.

Cartilage regeneration evaluation using second-look arthroscopy

All patients underwent second-look arthroscopy during hardware removal. Cartilage regeneration was evaluated macroscopically using the ICRS grading system in second-look arthroscopy^[40]. According to the ICRS grading system, grade I is considered normal, grade II considered nearly normal, grade III abnormal, and grade 4 severely abnormal.

Statistical analysis

Statistical analyses were performed using SPSS version 23.0 (SPSS, Chicago, IL, United States) with significance defined as $P < 0.05$. All data are presented as the mean \pm standard deviation. IKDC, WOMAC, and VAS scores were applied as the primary dependent variables in clinical outcomes. Wilcoxon signed-rank test was performed to compare the preoperative and postoperative state of articular cartilage in the patient cohort. Kruskal-Wallis test was performed to compare three or more variables. Mann-Whitney U test with Bonferroni adjustment was used for post-hoc comparison. Mann-Whitney U test was used to compare cartilage regeneration in patients with trochlear lesions *vs* patients without trochlear lesions. Simple regression analysis was performed to identify the effects of age and lesion size on clinical outcomes. The statistical methods used in this study were reviewed by Dr. Young Ju Kim from the Department of Statistics at the Catholic University of Korea.

RESULTS

In this study, 125 patients with an average age of 58.3 ± 6.8 years (range: 43-74 years old) were included, of whom 95 (76%) were female and 30 (24%) were male. The average body mass index (BMI) was 25.6 ± 2.7 kg/m² (range: 19.2-35.5 kg/m²) and average hip-knee-ankle (HKA) angle for measuring varus deformity was $7.6^\circ \pm 2.4^\circ$ (range: 5.0° - 14.2°). Seventy-three (58.4%) patients had trochlear lesions, while the remaining 52 (41.6%) did not (Table 1).

Second-look arthroscopic findings

Postoperative second-look arthroscopy with hardware removal was performed at 20.2 ± 6.5 mo (range: 8-38 mo) post-surgery. In second-look arthroscopy, the MFC cartilage status was as follows: 73 (58.4%) patients with ICRS grade I, 37 (29.6%) with ICRS grade II, and 15 (12%) with ICRS grade III. No patients had ICRS grade IV (Figure 4A-F) (Table 1).

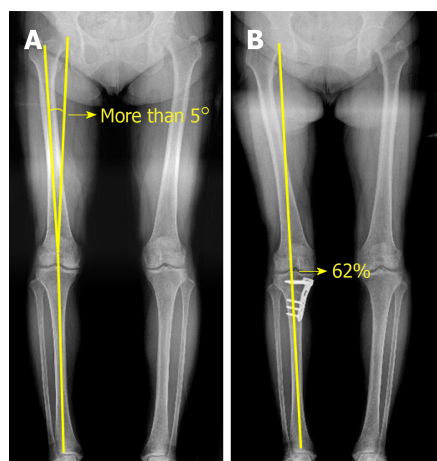


Figure 2 High tibial osteotomy. A: High tibial osteotomy was performed at a hip-knee-ankle angle of 5° or more; B: The mechanical axis was corrected to approximately 62% lateral to the tibial plateau.

Clinical outcome according to the ICRS grading system

Clinical results were analyzed by classifying patient groups according to the ICRS grading system. In the ICRS grade I group, the preoperative IKDC score was 28.4 ± 7.4 (range: 10.3–43.7) and increased to 59.3 ± 8.7 (range: 40.8–82.8), 64.3 ± 9.2 (range: 44.8–90.8), and 68.1 ± 10.8 (range: 40.2–90.8) at 1-, 2-, and 3-year follow-up, respectively ($P < 0.001$ for both 1- and 2-year follow-up; $P = 0.001$ for the final follow-up). The WOMAC score decreased from 45.1 ± 11 (range: 22–79) preoperatively to 11.0 ± 6.9 (range: 2–39), 8.4 ± 6.0 (range: 0–28), and 6.5 ± 6.0 (range: 0–33) at 1-, 2-, and 3-year follow-up, respectively ($P < 0.001$ for both 1- and 2-year follow-up; $P = 0.003$ at 3-year follow-up). The VAS score also decreased from 7.6 ± 1.4 (range: 4–10) preoperatively to 2.1 ± 1.7 (range: 0–7), 1.5 ± 1.4 (range: 0–6), and 1.1 ± 1.5 (range: 0–33) at 1-, 2-, and 3-year follow-up, respectively ($P < 0.001$ at both 1- and 2-year follow-up; $P = 0.004$ at 3-year follow-up).

All clinical outcomes in the ICRS grade I group improved significantly over time. In the ICRS grade II group, the IKDC score increased from 30.1 ± 7.2 (range: 16.1–54.0) preoperatively to 52.4 ± 10.7 (range: 29.9–70.1), 58.6 ± 11.1 (range: 40.5–82.8), and 61.0 ± 11.3 (range: 43–90.8) at 1-, 2-, and 3-year follow-up, respectively ($P < 0.001$ for both 1- and 2-year follow-up; $P = 0.063$ for 3-year follow-up). The WOMAC score decreased from 41.9 ± 9.2 (range: 30–64) preoperatively to 16.8 ± 8.5 (range: 5–40), 13.4 ± 8.2 (range: 1–39), and 10.5 ± 5.6 (range: 0–22) at 1-, 2-, and 3-year follow-up, respectively ($P < 0.001$ for 1-year follow-up; $P = 0.001$ for 2-year follow-up; $P = 0.002$ for 3-year follow-up). The VAS score decreased from 7.5 ± 1.1 (range: 6–10) preoperatively to 3.0 ± 1.6 (range: 0–7), 2.7 ± 1.8 (range: 0–8), and 2.0 ± 1.4 (range: 0–4) at 1- ($P < 0.001$), 2- ($P = 0.229$), and 3-year ($P = 0.019$) follow-up, respectively. In the ICRS grade III group, the IKDC score increased from 29.2 ± 8.1 (range: 11.4–43.6) preoperatively to 54.8 ± 7.1 (range: 45.2–70.1), 55.0 ± 8.0 (range: 40.4–75.9), and 59.3 ± 5.8 (range: 45.8–72.4) at 1-, 2-, and 3-year follow-up, respectively ($P = 0.001$, $P = 0.842$, and $P = 0.047$, respectively). The WOMAC score decreased from 44.3 ± 12.2 (range: 29–76) preoperatively to 17.6 ± 5.1 (range: 10–26), 17.3 ± 7.4 (range: 4–30), and 12.6 ± 8.3 (range: 1–28) at 1-, 2-, and 3-year follow-up, respectively ($P = 0.001$, $P = 0.607$, and $P = 0.018$, respectively). The VAS score decreased from 7.7 ± 0.8 (range: 6–9) preoperatively to 3.3 ± 1.2 (range: 1–6), 3.1 ± 1.2 (range: 1–5), and 2.6 ± 0.9 (range: 1–4) at 1-, 2-, and 3-year follow-up, respectively ($P = 0.001$, $P = 0.658$, and $P = 0.103$, respectively).

Preoperative scores showed no significant differences among the groups of patients (IKDC, WOMAC, and VAS; $P = 0.610$, $P = 0.275$, and $P = 0.817$, respectively). However, postoperative scores showed significant differences among patient groups at all the time points of follow-up (IKDC score: $P = 0.005$ at 1 year, and $P < 0.001$ at 2 and 3 years; WOMAC score: $P < 0.001$ at all follow-up time points; VAS score: $P = 0.002$ at 1 year, $P < 0.001$ at 2 and 3 years). Post hoc analysis revealed that except for IKDC at 1 year, all scores in the ICRS grade I group improved more than those of the ICRS grade II and III groups; IKDC scores of the ICRS grade II group did not differ significantly from those of the ICRS grade III group. The IKDC score of the ICRS grade I group differed significantly from that of the ICRS grade II group at 1-year follow-up; however, IKDC scores of other groups did not differ significantly at 1-year follow-up (Table 2).

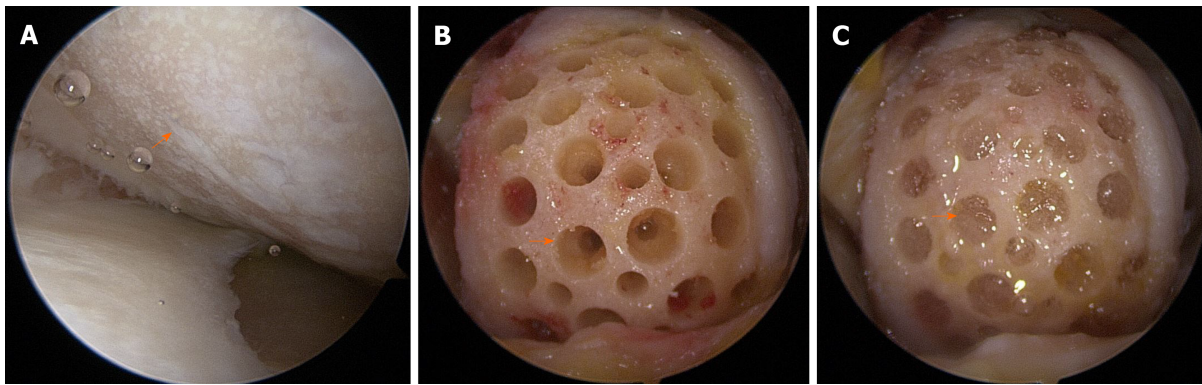


Figure 3 Arthroscopic findings of stem cell implantation procedures. A: Medial compartment osteoarthritis (arrow) in a 61-year-old woman; B: Multiple holes, 4 mm in diameter and 4 mm in depth (arrow), were drilled using a drill bit; C: Human umbilical cord blood-derived mesenchymal stem cells were mixed with hyaluronic acid hydrogel and implanted in the holes (arrow).

Clinical outcomes according to patient characteristics

Preoperative VAS scores differed significantly between the trochlear lesion group and the non-lesion group ($P = 0.046$); however, no significant differences in outcomes observed between these two groups at 1, 2, and 3 years postoperatively ($P > 0.05$ for all time points) (Table 3). Similarly, the WOMAC and IKDC scores did not differ significantly between the trochlear lesion group and the non-lesion group ($P > 0.05$ for all time points). The preoperative WOMAC score increased with increasing age ($P < 0.001$) but did not affect other outcomes ($P > 0.05$ for all). The lesion size of the MFC did not affect the IKDC score ($P > 0.05$ for all follow-up time points). The preoperative WOMAC score increased significantly with increased lesion size but did not affect the postoperative WOMAC score ($P < 0.001$ for preoperative WOMAC, and $P > 0.05$ for postoperative WOMAC). The VAS score increased significantly with increased lesion size at the preoperative stage ($P < 0.001$). However, there was no significant difference in postoperative outcomes ($P > 0.05$) (Table 4).

DISCUSSION

The results obtained in this study show that cartilage was regenerated to ICRS grade III or better in all cases after implantation of hUCB-MSCs with concomitant HTO. Jung *et al.*^[7] showed that cartilage was regenerated in MFC and MTP in second-look arthroscopy after medial opening-wedge HTO without any cartilage regeneration surgery. Although regenerated cartilage is mostly immature, we believe that reduced joint loading of the medial compartment after HTO provides an environment in which cartilage is regenerated. Accordingly, implantation or injection of MSCs with concomitant HTO has been used to enhance insufficient cartilage regeneration. Wong *et al.*^[23] investigated the injection of BM-derived MSCs with HA 3 weeks after MFx with HTO. They reported improved short-term outcomes, as well as magnetic resonance observation of cartilage repair tissue (MOCART) scores compared with those of the control group. Koh *et al.*^[24] compared a group treated with an injection of platelet-rich plasma (PRP) and concomitant HTO to a group treated with a dose of platelet-rich plasma (PRP), HTO, and an additional infusion of adipose-tissue-derived MSCs. Their results demonstrated that the group receiving MSC injection showed improved cartilage recovery and clinical outcomes compared with the group receiving a PRP injection only^[24]. Kim *et al.*^[22] confirmed that injection of adipose tissue-derived MSCs in 50 patients of MCOA improved clinical outcomes more than did HTO alone. Although we have not included a control group, we show that regenerated cartilage affected clinical outcomes in patients with varus deformity of more than 5° and full-thickness articular cartilage lesion of ICRS grade IV with more than 4 cm^2 in the medial compartment of the knee.

Regenerated cartilage in the ICRS grades I, II, and III groups improved the clinical outcomes of these patients, with the ICRS grade I group showing the best clinical outcomes among the three groups. Indeed, all scores in the ICRS grade I group improved over time compared with those of the ICRS grade II and III groups. These results indicate that cartilage regeneration *via* hUCB-MSCs implantation with concomitant HTO, is an effective approach to cartilage regeneration. In our present study, the regeneration status of articular cartilage in 73 (58.4%) patients, assessed *via* second-look arthroscopy, was judged to be ICRS grade I and accounted for the largest

Table 1 Patient demographic data

Patients	n = 125
Age, yr	58.3 ± 6.8
Sex, female/male	95 (76%)/30 (24%)
Lesion size, cm ²	6.9 ± 2
HKA angle, degree	7.6 ± 2.4
Trochlear lesion	
With lesion	73
Without lesion	52
Second look arthroscopic findings	
ICRS grade I	73
ICRS grade II	37
ICRS grade III	15

HKA: Hip-knee-ankle; ICRS: International Cartilage Repair Society.

proportion of the patients. Although some patients presented with partially regenerated cartilage, none showed a lack of cartilage regeneration (ICRS grade IV). We did not perform a histological examination to avoid damaging the regenerated cartilage in these patients. However, in patients with large chondral lesions, the regenerated cartilage fully covered the lesions, and showed adequate thickness and elasticity as assessed *via* palpation with a probe during second-look arthroscopy. Similarly, Park *et al*^[38] reported that hyaline-like cartilage was regenerated after hUCB-MSCs implantation in patients with OA, resulting in improved clinical outcomes in these patients.

Although the mechanisms involved in hUCB-MSC-mediated cartilage regeneration are only partially characterized^[46-48], it is clear that hUCB-MSC-based strategies are effective in treating patients with OA. Allogeneic hUCB-MSCs are not only standardized as off-the-shelf medicinal products, but also non-invasively yield a sufficient number of stem cells that can be applied according to the size of the cartilage lesion. Jo *et al*^[49] reported that patients with OA showed reduced pain levels and improved function after being treated with a high dose of adipose tissue-derived MSCs (1.0×10^8 cells) compared with a low (1.0×10^7 cells) or moderate dose (5.0×10^7 cells) administered intra-articularly. In addition, the cartilage defect area was regenerated into hyaline-like cartilage in the high dose group. However, the number of stem cells with respect to defect sizes has not been standardized and requires further investigation.

We also investigated whether patient age, presence of trochlear lesion, and size of lesion of MFC influenced clinical outcomes in patients with varus deformity. Several studies have shown that although OA is exacerbated by increased pressure in the patellofemoral (PF) joint after HTO, this process does not deteriorate clinical results or affect anterior knee pain^[50,51]. In our present study, patients with trochlear lesions showed significantly increased preoperative VAS scores compared to those without trochlear lesions; however, there were no differences in other scores between these two patient groups. The results of our present study show that implantation of hUCB-MSCs into the trochlea exerted a positive effect on cartilage regeneration. However, further studies are required to evaluate cartilage regeneration in the trochlea after hUCB-MSCs implantation with concomitant HTO. Furthermore, the preoperative WOMAC scores were the only variable affected by advanced patient age. Autologous sources of MSCs such as adipose tissue and BM are age-dependent^[52-54]; however, hUCB-MSCs maintain cell quality regardless as it is a cell therapy product. Finally, increased lesion size caused a subsequent increase in the preoperative VAS and WOMAC scores, but it did not affect other postoperative scores. These results indicate that implantation of hUCB-MSCs with concomitant HTO was applicable in patients with trochlear lesions and may even be a viable treatment option in patients with older or more extensive lesions.

Certain limitations were noted in this study. First, it was retrospective and did not include a control group. However, the presence of regenerated cartilage was confirmed *via* second-look arthroscopy in all the 125 patients. We also evaluated how the status of regenerated cartilage affected clinical outcomes. Thus, we can suggest the necessity of cartilage repair procedure during HTO. Second, although the presence of regenerated cartilage was confirmed visually and palpated using a probe, it was not

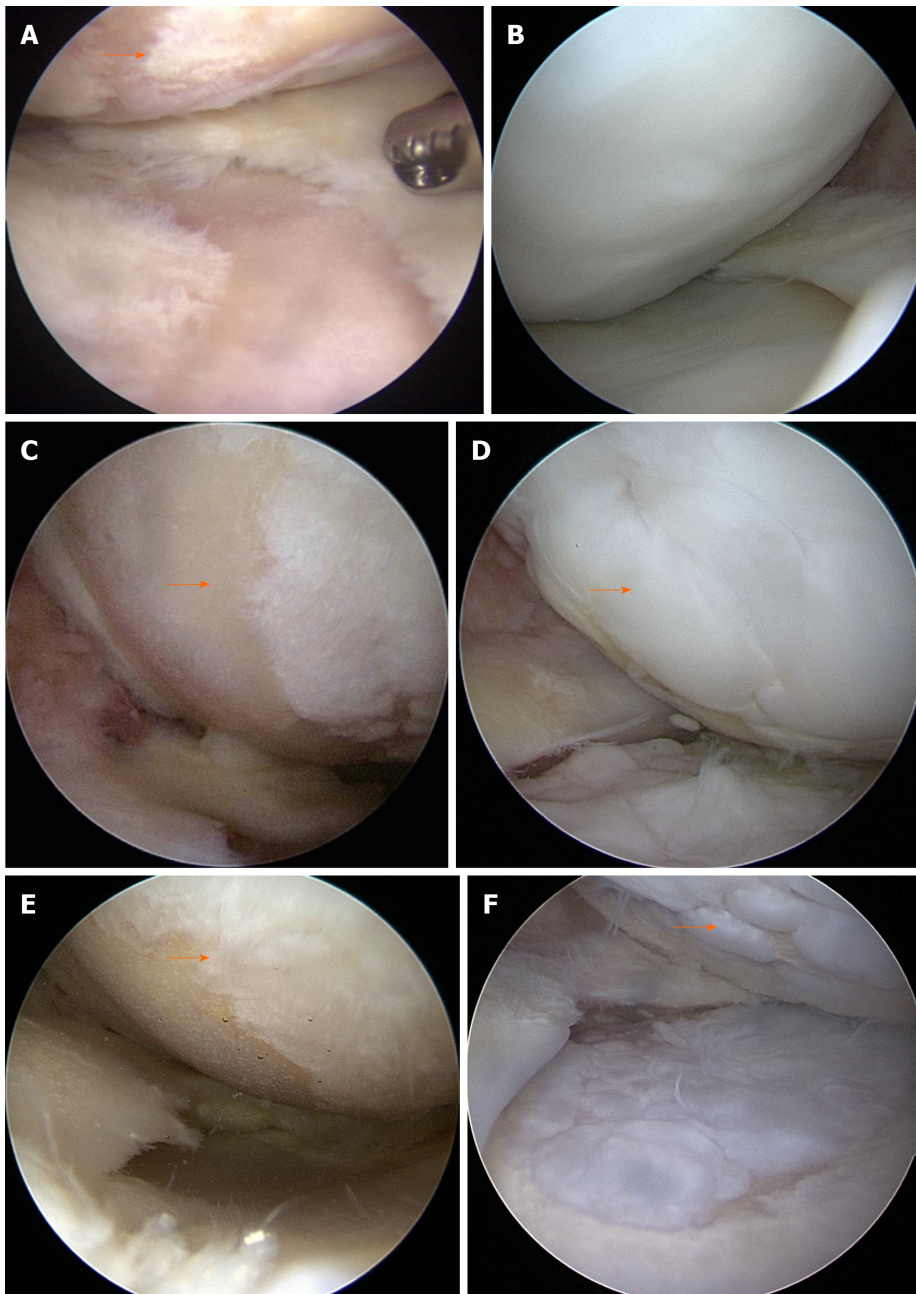


Figure 4 Second-look arthroscopic findings. A: Cartilage lesions classified as International Cartilage Repair Society (ICRS) grade IV in the medial femoral condyle (MFC) (arrow) and tibial plateau in a 61-year-old female patient; B: Cartilage was regenerated to ICRS grade I (arrow) ^{via} second-look arthroscopy, performed 13 mo after the initial operation; C: A cartilage lesion classified as ICRS grade IV in the MFC (arrow) of a 52-year-old male patient; D: Cartilage was regenerated to ICRS grade II (arrow) ^{via} second-look arthroscopy, performed 22 mo after the initial operation; E: A cartilage lesion of ICRS grade IV in the MFC (arrow) of a 68-year-old female patient; F: Cartilage was regenerated to ICRS grade III (arrow) ^{via} second-look arthroscopy, performed 16 mo after the initial operation.

evaluated histologically as that would have required a biopsy, which could damage the regenerated cartilage. Finally, a second-look arthroscopy was performed during hardware removal at an average time of 20.2 ± 6.5 mo post-surgery. This was a relatively short-term evaluation considering that cartilage remodeling requires an extended period of time. However, reducing the pressure in the medial compartment *via* HTO would preserve the regenerated cartilage and allow it to remain intact over time.

In conclusion, our results show that the implantation of hUCB-MSCs with concomitant HTO was an effective treatment option for patients with MCOA. We confirmed that regenerated cartilage improved clinical outcomes in this patient population. In addition, our results suggest that the presence of the trochlear lesions, the advanced age of the patient, or large cartilage lesions did not significantly affect clinical outcomes in patients with MCOA undergoing HTO with hUCB-MSCs implantation.

Table 2 Clinical outcomes according to the International Cartilage Repair Society grading system

	ICRS grade I (n = 73)	ICRS grade II (n = 37)	ICRS grade III (n = 15)	P value ¹	Post hoc ⁵
IKDC					
Preoperative	28.4 ± 7.4	30.1 ± 7.2	29.2 ± 8.1	0.610	
1 yr	59.3 ± 8.7	52.4 ± 10.7	54.8 ± 7.1	0.005	I > II = III, I = III
2 yr	64.3 ± 9.2	58.6 ± 11.1	55.0 ± 8.0	< 0.001	I > II = III
3 yr	68.1 ± 10.8	61.0 ± 11.3	59.3 ± 5.8	< 0.001	I > II = III
P value ²	< 0.001	< 0.001	0.001		
P value ³	< 0.001	< 0.001	0.842		
P value ⁴	0.001	0.063	0.047		
WOMAC					
Preoperative	45.1 ± 11	41.9 ± 9.2	44.3 ± 12.2	0.275	
1 yr	11.0 ± 6.9	16.8 ± 8.5	17.6 ± 5.1	< 0.001	I > II = III
2 yr	8.4 ± 6.0	13.4 ± 8.2	17.3 ± 7.4	< 0.001	I > II = III
3 yr	6.5 ± 6.0	10.5 ± 5.6	12.6 ± 8.3	< 0.001	I > II = III
P value ²	< 0.001	< 0.001	0.001		
P value ³	< 0.001	0.001	0.607		
P value ⁴	0.003	0.002	0.018		
VAS					
Preoperative	7.6 ± 1.4	7.5 ± 1.1	7.7 ± 0.8	0.817	
1 yr	2.1 ± 1.7	3.0 ± 1.6	3.3 ± 1.2	0.002	I > II = III
2 yr	1.5 ± 1.4	2.7 ± 1.8	3.1 ± 1.2	< 0.001	I > II = III
3 yr	1.1 ± 1.5	2.0 ± 1.4	2.6 ± 0.9	< 0.001	I > II = III
P value ²	< 0.001	< 0.001	0.001		
P value ³	< 0.001	0.229	0.658		
P value ⁴	0.004	0.019	0.103		

Boldface indicates statistical significance ($P < 0.05$).

¹Kruskal-Wallis test.

²Wilcoxon signed-rank test: Preoperative *vs* 1 year postoperatively.

³Wilcoxon signed-rank test: 1 year postoperatively *vs* 2 years postoperatively.

⁴Wilcoxon signed-rank test: 2 years postoperatively *vs* 3 years postoperatively.

⁵Bonferroni adjustment using the Mann-Whitney U test. ICRS: International Cartilage Repair Society; IKDC: International Knee Documentation Committee; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; VAS: Visual analog scales.

Table 3 Clinical outcomes according to trochlear lesion

	Trochlear lesion (n = 73)	No trochlear lesion (n = 52)	P value ¹
IKDC			
Preoperative	29.3 ± 7.3	28.5 ± 7.6	0.794
1 yr	56.7 ± 9.7	56.7 ± 9.7	0.960
2 yr	61.6 ± 10.7	61.4 ± 9.5	0.916
3 yr	64.7 ± 11	65.3 ± 11.4	0.493
WOMAC			
Preoperative	44.8 ± 10.3	43.1 ± 11.1	0.288
1 yr	13.8 ± 7.5	13.2 ± 8.2	0.471
2 yr	10.7 ± 6.9	11.3 ± 8.5	0.958
3 yr	8.4 ± 6.4	8.4 ± 6.8	0.755
VAS			
Preoperative	7.8 ± 1.2	7.3 ± 1.3	0.046
1 yr	2.6 ± 1.7	2.3 ± 1.6	0.331
2 yr	2.1 ± 1.5	2.0 ± 1.7	0.555
3 yr	1.6 ± 1.4	1.4 ± 1.6	0.203

Boldface indicates statistical significance ($P < 0.05$).

¹Mann-Whitney U test. IKDC: International Knee Documentation Committee; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index;

VAS: Visual analog scales.

Table 4 Effect of age and lesion size on clinical outcomes

	Age (<i>n</i> = 125)				Lesion size (<i>n</i> = 125)			
	β	<i>t</i>	<i>R</i> ²	<i>P</i> value ¹	β	<i>t</i>	<i>R</i> ²	<i>P</i> value ¹
IKDC								
Pre-op	-0.128	-1.429	0.016	0.156	-0.148	-1.661	0.022	0.099
1 yr	-0.095	-1.053	0.009	0.294	-0.025	-0.282	0.001	0.779
2 yr	-0.053	-0.593	0.003	0.554	-0.120	-1.339	0.014	0.183
3 yr	0.049	0.549	0.002	0.584	0.024	0.266	0.001	0.791
WOMAC								
Pre-op	0.327	3.831	0.107	< 0.001	0.305	3.550	0.093	0.001
1 yr	0.173	1.945	0.030	0.054	0.040	0.440	0.002	0.661
2 yr	-0.005	-0.055	< 0.001	0.956	0.052	0.575	0.003	0.566
3 yr	-0.048	-0.532	0.002	0.595	-0.064	-0.716	0.004	0.475
VAS								
Pre-op	0.115	1.286	0.013	0.201	0.335	3.943	0.112	< 0.001
1 yr	0.114	1.278	0.013	0.204	0.113	1.266	0.013	0.208
2 yr	0.001	0.013	<0.001	0.989	0.112	1.249	0.013	0.214
3 yr	-0.047	-0.519	0.002	0.605	0.164	1.842	0.027	0.068

Boldface indicates statistical significance (*P* < 0.05).¹Simple regression analysis. Pre-op: Preoperative; IKDC: International Knee Documentation Committee; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; VAS: Visual analog scales.

ARTICLE HIGHLIGHTS

Research background

High tibial osteotomy (HTO) is widely used to treat medial compartment osteoarthritis (MCOA) of the knee with varus deformity. HTO reduces knee pain and improves knee function by decreasing the pressure in the medial compartment of the knee.

Research motivation

HTO alone offers excellent short- and mid-term outcomes; however, these outcomes tend to deteriorate over time. For further improvement in knee joint condition, cartilage regeneration can be combined with HTO. Autologous chondrocyte implantation (ACI), osteochondral autologous transplantation (OAT), and microfracture have been known to be effective therapies for articular cartilage regeneration, but they are not suitable in case of osteoarthritis (OA) therapy. Recently, mesenchymal stem cells (MSCs) have been identified as a new option in the field of cartilage regeneration for the treatment of OA patients. The MSCs isolated from human umbilical cord blood (hUCB-MSCs) demonstrate higher proliferation and chondrogenic capacity than other MSCs. Reports on the clinical application of hUCB-MSCs are scarce, and there are no studies examining the use of hUCB-MSCs with concomitant HTO.

Research objectives

This study aimed to evaluate clinical outcomes and cartilage regeneration *via* second-look arthroscopy after implantation of hUCB-MSCs with concomitant HTO, for treatment of osteoarthritic knee with varus deformity.

Research methods

A total of 125 patients were included in this study with an average age of 58.3 ± 6.8 years (range: 43-74 years). All the patients had a varus deformity of more than 5° and a full-thickness International Cartilage Repair Society (ICRS) grade IV articular-cartilage lesion of more than 4 cm² in the medial compartment of the knee. All patients underwent second-look arthroscopy during hardware removal. Cartilage regeneration was evaluated macroscopically using the ICRS grading system in second-look arthroscopy. We also assessed the effects of patient characteristics, such as trochlear lesions, patient age, and lesion size, using the patients' medical records.

Research results

The results obtained in this study show that cartilage was regenerated to ICRS grade III or better in all the cases after implantation of hUCB-MSCs with concomitant HTO. Regenerated cartilage

in the ICRS grades I, II, and III groups improved the clinical outcomes of these patients. The ICRS grade I group showed the best clinical outcomes among the three groups. Indeed, all the scores in the ICRS grade I group improved over time compared with those of the ICRS grade II and III groups. Although some patients presented with partially regenerated cartilage, none of the patients showed lack of cartilage regeneration (ICRS grade IV).

Research conclusions

Our results show that implantation of hUCB-MSCs with concomitant HTO is an effective treatment option for patients with medial compartment osteoarthritis (MCOA). In addition, our results also suggest that the presence of trochlear or large cartilage lesions, or advanced age of the patient, does not significantly affect clinical outcomes in patients with MCOA undergoing HTO with hUCB-MSC implantation.

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