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EDITORIAL

Applications of single cell RNA sequencing to research of stem cells

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Abstract

Stem cells (SCs) with their self-renewal and pluripotent differentiation potential, show great promise for therapeutic applications to some refractory diseases such as stroke, Parkinsonism, myocardial infarction, and diabetes. Furthermore, as seed cells in tissue engineering, SCs have been applied widely to tissue and organ regeneration. However, previous studies have shown that SCs are heterogeneous and consist of many cell subpopulations. Owing to this heterogeneity of cell states, gene expression is highly diverse between cells even within a single tissue, making precise identification and analysis of biological properties difficult, which hinders their further research and applications. Therefore, a defined understanding of the heterogeneity is a key to research of SCs. Traditional ensemble-based sequencing approaches, such as microarrays, reflect an average of expression levels across a large population, which overlook unique biological behaviors of individual cells, conceal cell-to-cell variations, and cannot understand the heterogeneity of SCs radically. The development of high throughput single cell RNA sequencing (scRNA-seq) has provided a new research tool in biology, ranging from identification of novel cell types and exploration of cell markers to the analysis of gene expression and predicating developmental trajectories. scRNA-seq has profoundly changed our understanding of a series of biological phenomena. Currently, it has been used in research of SCs in many fields, particularly for the research of heterogeneity and cell subpopulations in early embryonic development. In this review, we focus on the scRNA-seq technique and its applications to research of SCs.

Key words: Stem cells; Heterogeneity; Single cell RNA sequencing; Developmental trajectories; Cell subpopulations

Core tip: Single cell RNA sequencing (scRNA-seq) has emerged as a powerful tool to

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explore cellular heterogeneity, provide new insights based on gene expression profiles of individual cells, reveal new cell subpopulations and predict developmental trajectories. It has been used in research of stem cells (SCs) in many fields, especially the study of heterogeneity and cell subpopulations in early embryonic development. This review aims to provide an overview of the applications of scRNA-seq to research of SCs.

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INTRODUCTION

Stem cells (SCs) are immature cells that are not fully differentiated. Based on the characteristics of self-renewal and pluripotent differentiation potential, SCs show great promise for widespread clinical applications, particularly to some refractory diseases such as stroke, Parkinsonism, myocardial infarction, and diabetes. Furthermore, as seed cells in tissue engineering, SCs have been applied widely to tissue and organ regeneration. Although the study of SCs has been ongoing for decades, there are still many issues to be resolved. One of the most striking phenomena is that even most SCs are homogeneous obviously within a single tissue, there are diverse subpopulations of cells showing unique distinct functions, morphologies, developmental statuses, or gene expression profiles compared with the other cell subpopulations[1-3]. Previous studies have indicated that the heterogeneity of cellular states is caused by the cell physiology, differentiation state[4,5], and its inherent plasticity[6-8], hindering further studies of biological characteristics and applications of SCs[9]. Although bulk-based approaches using microarrays of high throughput RNA sequencing (RNAseq) techniques provide certain important insights into SCs, these approaches are limited because results about structures and functions reflect average measurements from large populations of cells or the results are predominantly obtained from cells with superior numbers[10,11], overlooking unique biological behaviors of individual cells, conceal cell-to-cell variations. As a consequence, heterogeneity is still a major issue to be resolved in the research and applications of SCs.

Studies conducted at the single cell level are imperious to understand the heterogeneity of SCs. Although low throughput, single cell analysis techniques, such as single cell quantitative PCR, and single cell real-time quantitative PCR, have been used to test certain molecular markers of single cells, they are limited to studying small number of genes.

Based on the current technological advances in single cell technologies and the next-generation sequencing (NGS) approach, a new technique, single cell RNA sequencing (scRNA-seq), provides an effective measure to resolve the above mentioned issues[12]. scRNA-seq refers to whole transcriptome amplification at the single cell level, which comprises reverse transcription of mRNA into cDNA followed by cDNA amplification, and then high throughput sequencing. Compared with traditional sequencing techniques, scRNA-seq can efficiently describe heterogeneity of cell subpopulations, measure cell-to-cell variability of gene expression, identify previously unreported cell types and define associated cell markers[13,14], and describe developmental trajectories[1,15,16]. scRNA-seq has attracted much attention since its first discovery in 2009, and the applications to research of SCs grow continuously, particularly for the study of heterogeneity and cell subpopulations in early embryonic development[17,18]. Here, we discuss the scRNA-seq technique, its applications to research of SCs, and the future perspectives.

SINGLE CELL RNA CHALLENGES

Single cell isolation methods, whole transcriptome amplification at the single cell level, and high throughput RNA-seq have led to the development of modern scRNA-seq platforms. Current workflow of scRNA-seq is organized in a set of steps: Single cell isolation, reverse transcription of mRNA, cDNA amplification and sequencing library construction, high throughput sequencing, and computational analysis[19]. In

the steps above, isolating target single cells from a block of tissue or cultured cells is a critical step in scRNA-seq, which allows research at the single cell level. There are many approaches to isolate single cells, such as microfluidic systems, fluorescence-activated cell sorting, micromanipulation, and laser capture microdissection[20]. Compared with other isolation methods, microfluidic systems isolate and capture single cells in micron-scale channels, providing many advantages by allowing cell isolation from a small population, high throughput, reducing reagent costs, reducing pollution, and improving accuracy. This method can also be used to isolate rare cells. It provides a robust foundation for single-cell sequencing-based analysis, which is considered as an excellent method to isolate single cells[21,22]. Whole transcriptome amplification at the single cell level is critical for scRNA-seq and plays a significant role in producing adequate cDNA to construct the sequencing library. PCR-based methods, including degenerate oligonucleotide primed PCR, primer extension preamplification PCR, and ligation-mediated PCR, are common methods to achieve cDNA amplification. More recently, multiple displacement amplification and multiple annealing and looping-based amplification cycles, as advanced techniques in the field of single cell amplification have been found to produce a higher cDNA yield, higher fidelity, and lower amplification bias compared with PCR-based whole transcriptome amplification methods. The advancement of NGS technologies has facilitated single cell sequencing, enabling millions of DNAs to be sequenced simultaneously and allowing thorough analyses of genomes and transcriptomes. At present, prepared libraries undergo sequencing using high throughput RNAseq platforms including Fluidigm C1, DropSeq, Chromium 10X, SCI-Seq[23]. In terms of sequencing depth, the recent single cell transcriptomics sequenced 0.1–5 million reads per cell. To get saturated gene detection, 1 million reads per cell is generally recommended[19]. It is the rapid development of the three main technologies mentioned above that has increased the accuracy of scRNA-seq, extending its application and becoming a rapid focus of biomedical research. After high throughput sequencing, a comprehensive and systematic computational analysis is performed. In the current single-cell studies to decode heterogeneity in SC populations, methods conducted in this step include read quantification, quality control, dimension reduction and visualization of data, unsupervised clustering analysis, and differential expression analysis to interpret these acquired data sets. Researchers, who perform computational analysis, require knowledge of some programming languages, so that they can interact with the pre-established algorithms for aligning, clustering, and visualizing the data. Some specialized algorithms developed by advanced bioinformatics labs are in general used in unsupervised clustering analysis and differential expression analysis, such as DESeq2, MAST, and an easy to-use package, Seurat.

APPLICATIONS OF SINGLE CELL RNA SEQUENCING TO RESEARCH OF STEM CELLS

In the research of SCs, scRNA-seq is mainly used to identify cell subpopulations, analyze rare cell types, and describe developmental trajectories and regulatory networks.

Identification of cell subpopulations

One major application for scRNA-seq is research of stem cell heterogeneity. By acquiring unbiased samples of SCs from a tissue and generating transcriptomes for each cell, clustering cells is performed based on their expression data. Established clustering and dimension reduction methods, such as hierarchical clustering analysis, K-means, and principal components analysis, are usually applied to group cell subpopulations. The principle is that cells are sorted according to their expression levels of genes quantified by unique molecular identifiers. Cluster information is then overlaid on cells in two to three-dimensional t-distributed stochastic neighbor embedding plots that are used to visualize cell subpopulations. Performing statistical analysis to identify significantly differentially expressed genes between subpopulations to define cell markers assists in best discriminating different clusters, purifying, and distinguishing some cell subpopulations of interest. To functionally characterize the clustered subpopulations, functional annotation of differentially expressed genes is an indispensable step to analyze transcriptome data.

As an effective tool for research on heterogeneity of SCs, scRNA-seq is commonly applied to cancer SCs, adult SCs, and induced pluripotent SCs.

Cancer is regarded as one of the most complex and heterogeneous diseases, and cancer SCs are a major source for the formation, metastasis, and drug resistance of tumor. Intratumoral heterogeneity indicates a diverse pathological potential among

cancer SCs, which increases the difficulty in targeting therapy of cancer. Therefore, the heterogeneity of cancer SCs needs to be urgently addressed in cancer research, diagnosis, and treatment. The genetic information and differences in the expression and control of genes among individual cells can be detected by scRNA-seq, making it possible to understand intra-tumoral heterogeneity, map different clones in tumors, and analyze cancer SCs, which is informative for cancer research. Recent studies employing scRNA-seq for cancer research have investigated breast cancer[24,25], lung cancer[26,27], renal cell cancer[28], glioblastoma[29,30], and hepatocellular carcinoma[31].

Adult SCs, residinge in almost all tissues of the body, have a self-renewal capacity and multi-lineage differentiation potential under certain conditions. They are presently a research focus in the stem cell field. Among all types of adult SCs, adipose-derived mesenchymal stromal/stem cells (ADSCs) have received increasing interest for immune and hematopoietic modulation, anti-inflammation effects, pro-angiogenesis properties, and tissue repair and restoration, owing to their relative ease of harvest, abundance, and multi-lineage differentiation[32,33]. Numerous studies have demonstrated that ADSCs are heterogeneous populations consisting of various cell subtypes[34]. Accurately delineating subpopulations by functional properties or surface marker expression is necessary to promote their further translation to clinical benefits. Schwalie *et al*[35] revealed three distinct subpopulations of ADSCs and adipose precursor cells in subcutaneous adipose tissue using scRNA-seq. They demonstrated that one of these subpopulations, $CD142^+$ $ABCG1^+$cells, suppress adipocyte formation *in vivo* and *in vitro* in a paracrine manner. Furthermore, they showed that the mechanism of this action possibly involved Spink2, Rtp3, Vit, and/or Fgf12 genes. These findings suggested a potentially critical role for $CD142^+$ $ABCG1^+$ cells in modulating the plasticity and metabolic signature of distinct adipose cell-containing systems. Other studies on heterogeneity of adult SCs using scRNA-seq have investigated hematopoietic SCs[36] and neural SCs[37,38].

Induced pluripotent SCs are capable of unlimited self-renewal and can give rise to specialized cell types based on stepwise changes in their transcriptional networks. The research has indicated that gene expression is highly heterogeneous between induced pluripotent SCs, and the heterogeneity of cell states has not been described at a global transcriptional level. Nguyen *et al*[39] used scRNA-seq to study the heterogenous states of human induced pluripotent SCs represented in pluripotent cultures at the transcriptional level. Four independent subpopulations of cells were identified and defined. Next, cell trajectories of transition between pluripotency states were defined. In their study, the largest dataset of single cell transcriptional profiling of undifferentiated human induced pluripotent SCs was provided, which increased our understanding of the complexity of pluripotent SCs.

Analysis of rare cell types

The second area that benefits immensely from scRNA-seq is the analysis of rare cell types. Commonly used approaches, such as microarrays and the NGS approach of high throughput RNA sequencing, are limited to large populations of cells. In cases where samples are available in only trace quantities, each of which can have a distinct function and role, the transcriptome can hardly be profiled by sequencing using these techniques. scRNA-seq can be used to characterize hidden subpopulations of rare cell types and measure gene expression in individual cells, overcoming the limitation of the cell sample size during traditional transcriptome analysis. Although a limited number of cells can influence the results, it has been demonstrated that 30 cells is the minimum sample size to sufficiently analyze the complexity of large cell subpopulations[40]. In the early human embryo, only a very small number of embryonic cells and embryonic SCs can be isolated, which makes it difficult to study the gene regulatory network controlling human embryonic development by traditional methods. The problem has been solved by the development of scRNA-seq. Yan *et al*[41] analyzed 124 individual cells from human preimplantation embryos and human embryonic SCs at various passages using scRNA-seq. The number of maternally expressed genes was 22687, which was significantly more than 9735 maternal genes detected previously by cDNA microarray. The results provided a comprehensive framework of the transcriptome landscapes of human early embryos and embryonic SCs. Additionally, scRNA-seq is used in the research of trace quantities of cancer SCs.

Description of developmental trajectories and regulatory networks

Another important application of scRNA-seq is the description of developmental trajectories and identification of gene regulatory networks. Mapping the pathway of differentiation and elucidating the underlying molecular controls are major goals in the development of stem cell technologies. scRNA-seq can be used to study the molecular dynamics of various cell types during development, map developmental

trajectories, and reveal cell fate changes. During these processes, proliferative progenitor cells and stationary cells are detected, cell states that exist only transiently or during discrete time windows are identified, dynamic changes in the gene expression lineage of different cell types are recorded, and visualization of developmental trajectories is ultimately achieved. The application of SCs to the description of developmental trajectories and regulatory networks has been reported in many studies. Hematopoietic SCs, branching into all blood cell lineages of erythrocytes, leukocytes, and lymphocytes, must follow a highly controlled route. The molecular networks that control stem cell fate decisions, such as cell division or quiescence and differentiation or self-renewal, are still unclear. The chronological developmental trajectories of single hematopoietic cells from SCs to mature cells have not been described. Bendall *et al*[9] provided a comprehensive analysis of human B lymphopoiesis and constructed developmental trajectories from hematopoietic SCs through to naive B cells using scRNA-seq expression data, laying the foundation to apply this approach to other tissues. Muscle SCs activate, divide, and give rise to muscle progenitors when injuries occur. scRNA-seq was applied to capture the transcriptional state of individual muscle SCs and primary myoblasts. Dell'Orso *et al*[42] reported the homeostatic and developmental dynamic trajectories of regenerative adult muscle SCs and primary myoblasts, and described the relative transcriptional changes relative to metabolic pathways. In addition, other studies of developmental differentiation and gene regulation networks of SCs using scRNA-seq have focused on human pluripotent stem cell differentiation pathways[39], molecular trajectories of the early progenitors during human cord blood hematopoiesis[43], and developmental dynamics of adult hippocampal quiescent neural SCs[44].

In addition to the above applications to research of SCs, scRNA-seq can be used in the identification of cellular states such as the stage or speed of the cell cycle.

PERSPECTIVES

Recent progress in the development of scRNA-seq has been rapid and exciting. scRNAseq has enabled us to explore molecular profiles at the single cell level, which allows characterization of cellular heterogeneity and development. With the rapid development of scRNA-seq, many challenges have been encountered in the analysis, integration, and interpretation of single cell data. The limited efficiency of RNA capture and cDNA amplification bias may lead to distortion of gene expression profiles, which artificially magnify the cell-to-cell variability[3]. Numerous methods have been developed to essentially address these issues, such as optimization of protocols and improvement of computational and statistical methods. In summary, the past few years have witnessed remarkable growth of this technique, a trend we believe will continue, enabling deeper understanding of the biological complexity of SCs and related diseases.

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REVIEW

Genomic integrity of human induced pluripotent stem cells: Reprogramming, differentiation and applications

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Abstract

Ten years after the initial generation of induced pluripotent stem cells (hiPSCs) from human tissues, their potential is no longer questioned, with over 15000 publications listed on PubMed, covering various fields of research; including disease modeling, cell therapy strategies, pharmacology/toxicology screening and 3D organoid systems. However, despite evidences that the presence of mutations in hiPSCs should be a concern, publications addressing genomic integrity of these cells represent less than 1% of the literature. After a first overview of the mutation types currently reported in hiPSCs, including karyotype abnormalities, copy number variations, single point mutation as well as uniparental disomy, this review will discuss the impact of reprogramming parameters such as starting cell type and reprogramming method on the maintenance of the cellular genomic integrity. Then, a specific focus will be placed on culture conditions and subsequent differentiation protocols and how

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their may also trigger genomic aberrations within the cell population of interest. Finally, in a last section, the impact of genomic alterations on the possible usages of hiPSCs and their derivatives will also be exemplified and discussed. We will also discuss which techniques or combination of techniques should be used to screen for genomic abnormalities with a particular focus on the necessary quality controls and the potential alternatives.

Key words: Induced pluripotent stem cells; Genomic integrity; Mutations; Karyotype; Differentiation; Cell therapy; Quality control; Reprogramming

Core tip: The potential of human induced pluripotent stem cells (hiPSCs) is no longer questioned, with applications in many fields including disease modeling and cell therapy. However, the presence of mutations in hiPSCs is a concern. After a first overview of the mutation types currently reported in hiPSCs, this review is aimed at discussing the important points to understand and possibly control the occurrence of mutations during hiPSCs reprogramming, long term culture but also differentiation. Finally, the impact of genomic alterations on the possible usages of hiPSC derivatives will be discussed, with a focus on the necessary quality controls and the potential alternatives.

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INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) are artificial cells generated through complex genetic and epigenetic reprogramming of cultured somatic cells. They are close to human embryonic stem cells (hESCs) regarding their pluripotency, infinite self-renewal capacity but also when focusing on their genomic integrity. The first section of this review will describe the different types of genomic abnormalities reported in hiPSCs, ranging from large mutations involving wide karyotype alterations to single nucleotide mutations. Then, we will focus on the reprogramming process and its impact on the iPSC genome to discuss if reprogramming parameters can be adapted to minimize their effects on the cell genome. In a third part, we will focus on how iPSC genomic integrity is affected by both iPSC long term culture but also differentiation. Finally, the impact of genomic alterations on the possible usages of hiPSCs and their derivatives will be discussed as well as the necessary quality controls that need to be performed.

MATERIAL AND METHODS

This review is based on systematic research on PubMed (http://www.ncbi.nlm.nih.gov/pubmed) using the following research keywords (either used separately or in combination): "induced pluripotent stem cells, genomic integrity, and genomic stability". We prioritized the articles focusing on hiPSCs, and included those focusing on either hESCs or pluripotent stem cells from other species, including mouse, if they were particularly relevant in the context. We apologize for all the articles that we could not cite due to word limitations.

TYPE OF GENOMIC ABNORMALITIES OBSERVED IN HIPSCS: AN UPDATE

Karyotype aberrations

Although some cell lines maintain a normal karyotype after long-term culture,

hiPSCs, like hESCs, present a propensity towards genomic instability. Based on karyotype analysis using G-banding, a number of hiPSC lines present aneuploidies; including recurrent ones mainly acquired during long term culture such as trisomy of chromosome 12, 17 or X or amplification of specific locus. These abnormalities have been extensively reviewed[1-5]. It is now accepted that these chromosomal and sub-chromosomal aberrations are common features of both hESCs and hiPSCs, however it is unclear if this trend is a specific feature associated with pluripotency or whether it would also be observed in non-pluripotent cells hypothetically maintained in long term culture if possible. In addition to the more commonly known chromosomal abnormalities, an aberration known as uniparental disomy (UPD) has been reported in iPSCs[6,7]. UPD occurs when a daughter cell inherits two copies of a chromosome (or part of a chromosome) from one parental cell and no copy of the corresponding chromosome (or part of the chromosome) from the other parental cell. UPD can be associated with various clinical symptoms: it may lead to the acquisition of homozygosity of a recessive allele involved in a genetic disorder or to an imbalance of paternal versus maternal epigenetic information which may lead to dysfunction in case of the presence of imprinted genes[8]. For example, UPD of chromosome 15 leads either to Angelman Syndrome (if both copies of a section of chromosome 15 are obtained from the father) or to Prader-Willy Syndrome (if both copies are obtained from the mother), both serious developmental disorders. UPD has never been reported in the context of hiPSCs until recently when Bershteyn *et al*[6] used fibroblasts from patients affected with Miller Dieker Syndrome, a genetic disease characterized by the presence of a ring chromosome 17 and linked with congenital malformations. Authors reprogrammed these fibroblasts into hiPSCs using episomal vectors. Surprisingly, they showed that multiple hiPSC lines generated from these fibroblasts do not contain the ring chromosome. This was explained by the non-participation of the ring chromosome during reprogramming, leading to UPD of the whole chromosome 17. They showed the cell-autonomous correction of a ring chromosomal aberration via compensatory UPD by iPSC generation, opening the door to chromosome therapy using iPSCs. We also described within our laboratory that UPD could also be observed in hiPSCs in a non-compensatory context. One of the iPSC lines generated by repeated transfections using home-made mRNAs of normal human foreskin fibroblasts presented a complex and abnormal karyotype as well as a large region of UPD on the chromosome 1q[7]. Interestingly, we showed that despite the normal behavior exhibited *in vitro* in terms of stemness marker expression and the differentiation into cells from all three germ layers, this iPSC line exhibited an abolished ability to form teratoma *in vivo*. The potential link between these genomic rearrangements and this feature has not yet been elucidated. However, this observation demonstrated that UPD can also occur in hiPSCs in a non-compensatory context, even using a non-integrative reprogramming strategy. Moreover, this work highlights the importance of performing single nucleotide polymorphism (SNP) genotyping among the methods used for the quality control of hiPSC genomic integrity because UPD can only be detected by this method enabling an accurate detection of the regions with consecutive loci with loss of heterozygosity (LOH).

Copy number variations

Copy number variations (CNVs) are variations in the number of copies of DNA sections, consisting of either genomic sequence deletions or amplifications. The occurrence of CVNs in human pluripotent stem cells (hPSCs) was first highlighted in 2011. Laurent *et al*[9] performed an extensive analysis of 324 samples using high-resolution SNP genotyping. These samples included 37 hiPSC lines, 69 hESC lines, and non-pluripotent somatic cell lines or primary cell lines. The authors show that the number of CNVs in hiPSCs was significantly higher when compared to non-pluripotent samples. These results were confirmed in another study performed by Hussein *et al*[10] which analyzed 22 hiPSCs showing a higher level of CNV in hiPSC lines when compared to fibroblasts or hESC lines. The repartition of these CNVs is not random and they frequently affect common fragile sites or sub-telomeric regions, which can both be particularly sensitive to DNA double strand breaks. Two hypotheses may explain the presence of the high level of CNV in hiPSCs compared to hESCs or human somatic cell samples; either they are acquired *de novo* during the reprogramming process or they are pre-existing in the initial somatic cell population and are amplified or selected through reprogramming and subsequent culturing.

Single point mutations

Karyotyping, SNP genotyping or comparative genomic hybridization (CGH)-array analyses are techniques used to detect deletions or duplications in large parts of the genome, whereby each system has a specific detection limit (minimal size of a CNV detected) and resolution (genome coverage). However, these techniques are unable to

detect single point mutations, which can only be observed using sequencing. Through whole exome sequencing, Gore *et al*[11] analyzed the presence of single point mutations in 22 hiPSC lines and the 9 fibroblast populations they were derived from. The authors show that each iPSC line contained an average of 6 protein-coding mutations (*i.e.*, mutations in a coding region of the genome). The results have been confirmed by others; demonstrating the presence of between 6 and 12 single-nucleotide mutations of each human iPSC genome[12,13]. As noted with CNVs, there are two possibilities regarding the origin of these mutations: Are they preexisting in the initial population before reprogramming or acquired during reprogramming? The correct answer is most likely a combination of both, whereby its importance will be discussed in depth in section IIA. As such, another question remains; do these mutations offer a selective advantage for reprogramming or are they randomly amplified? Despite numerous debates, there is yet no consensus in the field and these two hypotheses are not mutually exclusive. On the one hand, various authors suggest that selection is possible as specific mutations have been found in at least 2 iPSC lines derived from the same fibroblast population or because these mutations frequently involve specific pathways[11,14]. On the other hand, other studies were unable to detect such 'shared' mutations and therefore do not support this hypothesis[12].

THE IMPACT OF REPROGRAMMING: FINDING THE KEY TO GENOMIC INTEGRITY?

The existence of mutations in iPSCs is currently well established. However, the subsequent challenge is to try to understand whether such genomic abnormalities could be reduced or minimized. Several aspects have been highlighted as potential factors involved in maintaining or compromising the iPSC genome integrity and will be discussed in the following section.

The importance of somatic mosaicism; choosing the right cell type to reprogram

The importance of somatic mosaicism (the coexistence of cells with different genotypes in a cell population) in the context of iPSCs has been demonstrated in a study focusing on Down syndrome [resulting from chromosome 21 trisomy (Ts21)]. In rare cases (1%-3% of patients), patients are mosaic for this mutation whereby only a percentage of their cells carry the trisomy. In this study, authors used mosaic patient's fibroblast population with 90% of the cells carrying the Ts21, whereas the remaining 10% of cells were euploid. They subsequently generated 3 iPSC lines using the fibroblast population and demonstrated, through fluorescence *in situ* hybridization analysis, that two cells lines contained Ts21, whilst one cell line was euploid for chromosome 21, highlighting the clonogenic characteristic of reprogramming and its subsequent impact on iPSC genome[15]. Authors also performed SNP analysis and excluded the possibility of UPD, which may have explained a trisomy rescue[15].

This example highlights the importance of considering somatic mosaicism as a crucial parameter to take into account when ensuring the maintenance of hiPSC genomic integrity, as iPSC generation involves the cloning and amplification of the genome of one unique cell. Somatic mosaicism accumulates during mitosis and is therefore acquired both during early development and during the normal aging process. It has been shown to affect various tissues such as skin, cerebellum, liver, intestine or digestive tract, and depends on the tissue self-renewal rate and exposure to environmental stress such as ultraviolet radiation[16,17] or endogenous mutagenic factors such as transposable elements[18]. Since such events accumulate with ageing, donor age has been shown recently to be associated with an increased risk of abnormalities in iPSCs[19]. The definition of somatic mosaicism also includes genomic alterations of varying size, ranging from chromosome gains or losses to single nucleotide substitutions. A number of studies have focused on the genomic integrity of iPSCs, highlighting the contribution of somatic mosaicism, either through the acquisition of CNVs or single point mutations. Abyzov *et al*[20] analyzed 20 hiPSC lines generated from 7 different fibroblast populations. They showed that each iPSC line contained an average number of 2 CNV (< 10 kb). Using both polymerase chain reaction (PCR) performed across CNV breakpoints and droplet digital PCR, the authors illustrate that at least 50% of the CNVs detected in the hiPSC lines were present at a very low frequency in the original fibroblast population; and therefore can be explained by somatic mosaicism. It should be noted that the value obtained (50%) may be an underestimation, depending on the detection level of the technique used and the quantitative contribution of the CNV[20]. The authors analyzed the 7 populations of fibroblasts and showed that 30% of them contained CNVs when compared to a human genome reference sequence such as hGRC37 sequence,

highlighting a high degree of somatic mosaicism in fibroblasts. Investigations focusing on single point mutations, specifically protein-coding mutations, have also underlined the contribution of somatic mosaicism in iPSC line genetic abnormalities; however the quantitative estimation differs from one study to another. One study describes a total average number of 6 protein-coding mutations per hiPSC genome and the authors then quantified the frequencies of these mutations in the corresponding fibroblast lines using ultra deep sequencing and showed that approximately 53% of the mutations were found in the original fibroblast lines; ranging from 0.3-1000 in 10000[11]. These conclusions have been further supported by another study showing that at least 17% of protein-coding mutations in hiPSCs can be detected in the originating fibroblast population[13]. Moreover, using Next Generation Sequencing on both iPSC clones and fibroblast subclones they were derived from, Kwon *et al*[21] highlighted that only a small number of variants remained undetectable in the parental fibroblasts. This data has also been reinforced in the mouse model through a study demonstrating that different murine iPSC lines share SNP variants; therefore suggesting that these mutations are present in a subpopulation of the fibroblasts[14]. The existence of somatic mosaicism also poses the question of whether it is necessary to generate isogenic controls when using iPSCs for disease modeling. To date, "normal" iPSCs, cells derived from an unaffected individual, are often taken as a control for pathological iPSCs. However, considering the importance of the genetic background of each iPSC line, the optimal control would be an isogenic iPSC line. These cell lines can either be generated by specifically targeting the mutation in the affected iPSC line using recently developed genomic editing strategies (CRISPR/Cas9 or TALENs)[22] or be generated by chance; as reported in the case of the Down Syndrome study where the euploid derived iPSC line could be used as the optimal isogenic control to study the physiopathology of the disease[15].

Based on these conclusions, the next question to address is "Can we reduce the contribution of somatic mosaicism by using a specific cell type, and thus improve the control of genomic integrity in iPSCs?" Unfortunately, the answer is still not clear. Various cell types have been shown to be susceptible to reprogramming including fibroblasts, keratinocytes, mesenchymal stem cells, blood cells, hepatocytes *etc*. However, little is known about the extent of somatic mosaicism in the different cell types and comparative genomic analyses are not currently available. However, two cell types, blood-derived cells and urine-derived cells, have been found to be suitable cells for use in reprogramming, with an added advantage of being easily obtained. In a first article, authors isolated endothelial progenitor cells (EPCs) from peripheral blood followed by successful reprogramming into iPSCs using retroviral vectors. The team performed karyotype and CGH-array analyses and did not detect any genomic abnormalities were detected in 9 of the 11 EPC-iPSC lines. The remaining two EPC-iPSC lines were shown to have one copy gain of 36.6 and 632.7 kb in size, respectively[23]. Previous studies have shown that higher numbers of CNVs are detected when using fibroblasts as the initial substrate from reprogramming[10,24,25], therefore the authors suggest that EPCs, which are easily isolated and present a relative immature phenotype, could be used to generate genetically healthy iPSCs. In another article, the authors reprogrammed human cord blood (CB) CD34+ cells using lentiviral vectors. Through whole exome sequencing analysis of 5 iPSC lines, an average of 1.3 coding mutations per iPSC line was detected, which is lower when compared to previous studies using the same analysis technique[26] although CB is not an optimal source based on accessibility in the context of personalized medicine. However, a direct comparison of both substrates in comparable conditions such as iPSCs generated in parallel with the same reprogramming methods, culture conditions, genomic analysis techniques and detection criteria, would be needed to confirm these results. Nagaria *et al*[27] showed that hiPSCs derived from CB myeloid progenitors closely resembled hESCs in DNA repair gene expression signature and irradiation-induced DNA damage response, relative to hiPSCs generated from CB or fibroblasts via standard methods. Another cell type of interest is urine-derived cells. Since the first proof of concept in humans[28], it has now been shown that human iPSCs can be successfully generated using urine-derived cells in xeno-free conditions[29]. However, apart from the absence of genomic integration after episomal reprogramming and the conservation of a normal karyotype, there is no additional data on the genomic integrity of these cells. Thus, an extensive study of the different cell types relating to the incidence of somatic mosaicism would be highly beneficial.

The method of reprogramming

It is well known that the integration of a viral cassette into the genome is directly linked to a risk of insertional mutagenesis[30]. Therefore, in an attempt to overcome this issue, a number of teams have focused on the development of non-integrative reprogramming methods over the last few years, in order to bridge the gap for the use

of iPSCs in a clinical setting. Despite the fact that the use of non-integrative reprogramming methods will be a prerequisite in the future, only a few articles report the analysis of the impact of the reprogramming method on iPSC genomic integrity. Initially, focusing on single-nucleotide coding mutations detected by exome capture sequencing, Gore *et al*[11] did not observe a link between the reprogramming method and the number of protein-coding mutations. The study investigated the impact of three different integrative methods and two non-integrative methods; using a total of 22 iPSC lines. This investigation pioneered the quantification of genomic integrity in hiPSCs. However, one limitation of this study was the use of various hiPSC lines from different laboratories (with each laboratory having its own culture methods) and therefore cannot be regarded as a strict comparison between the different reprogramming techniques. Another large cohort was analyzed by Hussein *et al*[10]. The authors analyzed 22 hiPSC lines generated within their laboratory either through retroviral transduction or piggyBac gene delivery methods. Using Affymetrix SNP array, the authors found approximately 109 CNVs per iPSC line (minimal size 10 kb, 10 markers). Once again, the study showed that the delivery method of the reprogramming factors did not influence the resulting data. On the other hand, there are a few articles that highlight the potential impact of the reprogramming techniques using a smaller cohort of hiPSC lines. Cheng *et al*[12] analyzed three hiPSC lines generated by episomal reprogramming of blood-derived CD34+ cells or MSCs. The authors carried out whole genome sequencing as well as CNV analysis and observed 6 to 12 coding mutations per iPSC line, reinforcing previously published data[11], and demonstrated the complete absence of CNV in the three iPSC lines[12]. In another article, Boreström *et al*[31] successfully reprogrammed both human foreskin fibroblasts and primary chondrocytes using the mRNA reprogramming system provided by Stemgent, which was based on the work carried out by Warren *et al*[32]. They performed both karyotype and CNV analysis by Affymetrix SNP 6.0 array and observed that all the iPSC lines generated are free of acquired CNV[31]. However, the minimal size of CNV detection and the criteria used for detection have not been indicated, furthermore additional whole genome sequencing or exome sequencing would be necessary to fully confirm the development of a "footprint-free" iPSC generation strategy. Due to the importance of addressing this issue, our team wanted to assess the genomic integrity of iPSC lines that were generated using repeated transfections of mRNAs. We also analyzed iPSCs generated from retroviral transduction as a comparative control. All the analyzed hiPSC lines originated from the same fibroblast population and were cultured in the exact same conditions. Using SNP analysis, we demonstrated that mRNA-derived iPSCs do not significantly differ from the parental fibroblasts in SNP analysis, whereas significant differences were noted when comparing retrovirus-derived iPSCs and the parental fibroblasts. On the other hand, CNV analysis confirmed that the number of CNVs may not be dependent on the reprogramming method itself, but instead appeared to be clone-dependent[33]. The first evidence demonstrating the link between the number of CNV and the reprogramming method has been made in a mouse model. Park *et al*[34] reprogrammed murine primary hepatocytes using either a polycistronic vector (lentiviral or retroviral transduction of OKSM factors) or through repeated delivery of purified recombinant proteins. CNV analysis was then performed using a custom 1M array CGH platform on 10 iPSC lines, at passage 18. The authors showed an increase in CNV content in the lenti-miPSC and retro miPSC lines which had from 29 to 53 CNVs depending on the cell line, compared to protein-miPSC lines (from 9 to 10 CNVs)[34]. Due to the costly and labor intensive nature of generating hiPSCs using different reprogramming strategies in comparable conditions, in addition to the financial resources and expertise's required to perform high quality genomic analysis, limited data exists demonstrating the various impacts of non-integrative reprogramming strategies versus integrative methods. Addressing these issues, an extensive study was recently published. The authors compared 3 different non-integrative reprogramming methods (mediated either by mRNA, sendai-virus or episomes) and 2 integrative reprogramming methods (lentivirus-mediated or retrovirus-mediated). Several parameters were analyzed such as reprogramming efficiency, success rates, labor intensity etc. Karyotype and CGH-array analyses were used to investigate the effects on hiPSC genomic integrity. Based on karyotype analysis of representative iPSC lines, the percentage of aneuploid iPSC lines generated was significantly lower (2.3%) for mRNA-iPSCs when compared to retrovirus (8.3%) or Epi-hiPSC (11.5%), a positive advantage for using the mRNA strategy[35]. The authors also found that the majority of CNVs are preexisting in the fibroblast population and that the frequency of *de novo* CNVs was particularly low in all iPSC lines and no link between the reprogramming method and the number of CNVs was highlighted, reinforcing the conclusion drawn in our laboratory and others. Another study later confirmed these results comparing mRNA, retroviral and sendai- reprogramming strategies and showed only subtle

differences among the methods, with most of the detected variants also reported among the fibroblast population[36]. In contrast, other studies reported that the number of CNVs and cytogenetic rearrangement in the genomes of the integrating iPSC lines were 20 and 7 times higher than those of the non-integrating iPSC lines, respectively[37,38].

Taken together, initial conclusions of these studies highlight the fact that no method has a zero impact on iPSC genomic integrity, despite the positive advantage of non-integrative reprogramming methods compared to integrative ones. Further investigation, including an extensive analysis using whole-genome sequencing, is required to fully understand the benefits of one reprogramming strategy when compared to another with regards to maintaining iPSC genomic integrity. Indeed, even non-integrative reprogramming requires extensive analysis of genomic integrity of the resulting iPSCs, combining methods that enable the detection of large rearrangements (karyotype analysis) including UPD (SNP analysis), deletions or duplications (CGH array or SNP analysis) and single point mutations (sequencing), especially when aiming at utilizing such cells for therapeutic applications.

The impact of other parameters on the genomic stability of hiPSCs

Once the reprogramming strategy has been defined, specifically the choice of the starting cells and the reprogramming method, it is important to identify other parameters that have been shown to impact the genomic integrity of iPSCs. Chen *et al*[39] highlight a potential dosage effect of the reprogramming factors on the occurrence of CNVs in iPSCs. The authors analyzed 41 mouse iPSC lines generated from the same parental donor. Varying combinations of the reprogramming factors (the experiments were performed using high-performance engineered factors versus normal reprogramming factors) and various concentrations of reprogramming factors were investigated. Using CGH-array, the authors show that rates of CNVs were negatively correlated with the concentration of the classic Yamanaka factors and that the use of high-performance factors also lead to a significant reduction in the CNV number. In parallel, the use of high reprogramming factor concentration and high-performance factors led to higher number of clones and reduced the time for the first colonies to appear, suggesting a direct relationship between the reprogramming efficiency/strength and the genomic integrity of the iPSCs[39]. Sugiura *et al*[40] showed that these reprogramming-associated mutations arise during the initial stages of the conversion of these cells. It should also be noted that the culture conditions, in particular media composition, may also play a role in maintaining iPSC genomic integrity. Ji *et al*[41] showed that supplementing the reprogramming media with antioxidants could reduce the genomic aberrations within the hiPSCs. Utilizing NAC (N-acetyl-cysteine) treatment followed by SNP analysis, the authors were able to significantly reduce the number of CNV by 3.9-fold (12 CNVs versus 47 for the non-treated cells). However, due to the high variability between the results, single point mutations analyzed by high-throughput genome sequencing did not show any defined trends and the mechanism behind the CNV number reduction is not clear. Another group, Luo *et al*[42], used either a commercial antioxidant or a home-made cocktail of three antioxidant molecules (L-ascorbate, L-glutathione, and α-tocopherol acetate) and demonstrated that the in-house cocktail had a protective effect on one of the two hiPSC lines used. On the other hand, no obvious changes were observed with the use of the commercial product. Mechanistically, the induction of reprogramming factors within cells leads to an acceleration of the growth rate and therefore a higher metabolic demand. In this view, Lamm *et al*[43] highlighted that accumulating aneuploid hPSCs undergo DNA replication stress, resulting in defective chromosome condensation and segregation. Compared to mouse ESCs and fibroblasts, mouse iPSCs had lower DNA damage repair capacity after a specific ionizing radiation performed to induce double strand breaks[44]. Moreover, repair mechanisms seems to lose efficiency during long-term passaging of hiPSCs[45]. However, this finding has been challenged in a study showing that mouse iPSCs, compared to mouse ESCs and mouse differentiated cells, showed enhanced resistance to mutagenesis with higher level of base excision repair proteins[46]. Esteban *et al*[47] showed that reprogramming is directly associated with increased levels of reactive oxygen species within the cells, which may then lead to single and double-strand DNA breaks; a major cause of genomic transformations. As an illustration, strategies aimed at limiting the reprogramming-induced replicative stress by either increasing checkpoint kinase 1 levels or the adding nucleoside supplements to the culture media has been shown to have a protective effect on DNA damages during reprogramming[48,49].

In conclusion, based on the results from the various investigations, it can be suggested that iPSCs are likely to suffer from genomic instability during the reprogramming process which is directly related to the efficiency of the reprogramming technique. In other words, effective robust reprogramming technique

will generate iPSCs with a significantly reduced number of genomic alterations. Hence, it is reasonable to assume that our efforts should now focus on increasing the efficiency of reprogramming as a whole. Furthermore, these conclusions have recently been reinforced in the mouse model. The authors used selected small molecules (PD0325901, SB431542, thiazovizin and ascorbic acid) combined with retroviral reprogramming and showed that, in addition to promoting rapid and efficient reprogramming of mouse fibroblasts, this cocktail of small molecules acts to stabilize genomic integrity (karyotyping analysis) through the activation of the Zscan4 (zinc finger and SCAN domain containing 4) gene and facilitation of the DSBs repair[50].

MAINTAINING GENOMIC INTEGRITY DURING CELL CULTURE AND DIFFERENTIATION

Culturing hiPSCs: Can these cells be pampered?

We have discussed in detail the various factors that can impact the genome integrity of iPSC during the reprogramming procedure. However, specifically with regards to clinical applications, the final product is not the iPSC line itself but a differentiated progeny. PSCs are able to self-renew indefinitely *in vitro*, through regular manual passaging (commonly performed once to twice a week for human PSCs) to obtain a sufficient number of cells for further characterization and differentiation assays. A few years ago, human iPSCs were first used in clinics to treat age-related macular degeneration. In this case, approximately 5×10^5 iPSCs (easily obtained in culture) were required to generate a hiPSC-derived retinal pigmented epithelium (hiPSC-RPE) sheet with a diameter of 1 cm (sufficient to cover a macular area with a 3 mm diameter)[51]. However, the use of iPSCs in other applications such as myocardial injury and non-human primate heart transplantation, require the delivery of 1×10^9 cells[52], therefore, from single-colony selection, several successive passages are necessary to obtain a sufficient amount of hiPSCs. Since 2004, it is commonly accepted that culturing hESCs long time is directly associated with classical aneuploidies such as trisomy of chromosome 12, 17 and X, or sub-chromosomal aberrations in chromosome 20 for example[53-57]. These aberrations have also been frequently reported in hiPSCs and have been shown to confer specific growth advantages such as the recurrent trisomy of chromosome 12p which contains the *NANOG* gene involved in cell pluripotency, trisomy of the chromosome 17q including genes like *SURVIVIN* or *STAT3* linked to self-renewal[55], or 20q11.21 duplication being linked to genes with anti-apoptotic effects[56,58]. Mayshar *et al*[59] demonstrated that these aberrations, previously detected by CGH-array, could also be identified using a gene expression analysis platform. The technique is based on the knowledge that biased gene expression is directly correlated with such chromosomal abnormalities, enabling a retrospective, albeit less sensitive, examination of iPSC genomic integrity. Laurent *et al*[9] revealed a trend among CNV apparition describing the recurrent deletions of tumor suppressor genes at early passages and the duplication of oncogenes at late passages. Moreover, analyzing both hESC and hiPSC lines, Hussein *et al*[10] concluded that long-term passaging is associated with a decrease in both the CNV number and the total size of CNVs. With regards to iPSCs, the majority of the CNVs generated during the reprogramming process (either selected or acquired) disappeared after 30 passages. The authors suggest that DNA repair would not be sufficient to explain this phenomena and hypothesize that hiPSC endure a bidirectional CNV selection, both against and in favor of CNVs present at early passages, where the rate of the selection pressure is more important during the first set of passages. In addition to active selection, *de novo* CNVs could also be acquired at late passages[10]. A recent study performed on 140 independent hESCs lines highlighted recurrent dominant negative *TP53* mutations, with the mutant allelic fraction increasing with passage number, suggesting that these mutations confers selective advantage to the cells[60]

During long term culture, it can be assumed that the act of passaging itself creates a stress factor that in turn may induce genomic instability. Various reports suggest that enzymatic passaging may lead to cytogenetic aberrations[57,61,62]. Furthermore, additional investigations have demonstrated that mechanical passaging is also associated with cytogenetic abnormalities[63,64]. The first systematic analysis of the impact of the passaging method was performed by Bai *et al*[65] using 3 different hESC lines. Through karyotype analysis and CNV detection using SNP genotyping, the authors first analyzed the number of CNVs present in these cell lines at passage 13 (considered as P0 for the temporal analysis). They showed that hESCs that were subsequently passaged, post p13, using enzymatic dissociation (TrypLE + Rho-kinase inhibitor Y27632) rapidly acquired supplemental CNVs (within 5 passages) in comparison with mechanical passaging where the number of CNVs remained stable

over 10 passages, and up to 30 passages for one of the three cell lines tested. The authors also demonstrated that single cell passaging, induced by enzymatic dissociation, was associated with increased DNA double strand breaks which in turn could be regarded as a cause of generating the CNVs observed. The study suggested that these abnormalities did not exist in the iPSCs prior to their comparative analysis (based on a mathematic model taking into account the cell population doubling rates) and are therefore induced by the enzymatic passaging itself. The authors also showed that this effect is not due to the presence of ROCK inhibitor in culture. It can reasonably be assumed that these findings are also applicable to hiPSC lines, and in order to optimize their use in clinical applications, the culture time should be maintained at a minimum. Recently, a longitudinal study was published combining CNV analysis and the culturing of 3 hiPSC lines and one hESC line for a highly extended period of time (2 years). Using four selected combinations of culture and passaging conditions, the authors reported that enzymatic passaging on a feeder-free substrate was associated with an increased accumulation of genetic aberrations compared to mechanical passaging on feeder layers[65]. They also show that the passaging method has a stronger effect when compared to the substrate, reinforcing previously cited results[66]. Besides the passaging method, another study focusing on hESCs highlighted a correlation between cell culture density and the occurrence of DNA damage and genomic alterations, likely triggered by medium acidification due to increased lactate concentrations in high density cultures. In this view, increasing the frequency of media changes restores the DNA damage to its basal level[67]. However, 3D culture systems may rapidly replace conventional monolayer growth systems and various reports have demonstrated that hPSCs may be expanded long-term using scalable 3D suspension culture systems[68], in chemically defined and xeno-free conditions. One article revealed that hESCs cultured in these conditions retained a normal karyotype for over 20 passages[69]. However, the potential protective effect of such a culture system on iPSC genomic integrity is yet to be further investigated.

Another component of the reprogramming system is the oxygen percentage in the incubator where the cells are cultured. The expansion of iPSCs is commonly performed under 5% CO_2; 20% O_2 incubator (normoxia). However, this oxygen condition differs from the one present in the physiological "niche" of pluripotent stem cells found in the embryo inner cell mass, which is in hypoxia[70]. iPSCs, by definition, do not have physiological niches and can therefore be generated both in normoxia and hypoxia. Various articles suggest that hypoxia improves reprogramming efficiency[31,71,72] and can induce re-entry of committed cells (spontaneous differentiated cells from ESC culture) into a fully pluripotent state[73]. The precise temporal role of hypoxia during human reprogramming has been recently studied[74]. In addition, a study demonstrated that the expression of the MMR (DNA mismatch repair), which normally corrects replication errors, was down regulated in both mouse neural stem cells and human mesenchymal stem cells exposed to hypoxia[75]. This malfunction is also involve in the genomic instability of several tumors[76]. Finally, the MMR defect may partially explain why hypoxia (5% O_2) is able to increase reprogramming efficiency, which is also the case with p53 inhibition, but likely at the cost of genomic integrity[77]. However, there is no strong evidence showing the potential effect of long-term low-oxygen culture conditions on the maintenance of genomic integrity despite the obvious limited oxidative stress in hypoxic conditions.

Last but not least, the culturing of iPSCs prior to differentiation could include a step of genetic correction, in the case of personalized iPSC-based therapy where the patient's iPSCs carry a particular pathogenic mutation. In this respect, recent advances have been made using genome editing technologies on hPSCs (including TALEN and CRISPR/CAS9 systems[78,79], enabling the precise targeting of specific sequence in the genome. However, potential off-target modifications of the genome have been reported[80] and will have to be carefully assessed to ensure maximum gene targeting efficiency and specificity[81]. Moreover, these genome editing strategies often imply selection of a corrected single cell-derived clone through selection pressure relying on the expression of a gene allowing drug resistance. Again, this selection favors accumulation of genetic damage.

The effects of differentiation on hPSC genomic integrity

The directed differentiation of hPSCs into functional terminally differentiated cells is now possible due to the availability of matrices, cytokines and growth factors required to drive the differentiation process. Depending on the specific cell type, hPSC differentiation can be a long and arduous process for the cells, whereby their stemness characteristics are lost and with time are replaced by the morphology and functional properties associated with the differentiated cell type. During human development, such differentiation processes take several weeks or months, however in vitro specific protocols try to recapitulate the process in a significantly shorter

period of time. Considering the significant metabolic and epigenetic changes required in undergoing such a transition, the following question arises: how does the differentiation process affect the genomic integrity of the pluripotent stem cells? Despite its importance when considering the use of the differentiated hPSCs in therapeutic applications, there are a limited number of published studies investigating this aspect. One study differentiated six hESC lines into neural stem cell populations which could be propagated *in vitro* for over 50 passages without entering senescence. The researchers showed that this particular property was associated with a jumping translocation involving chromosome 1q. The analysis was performed after the long-term culture of these derivatives (at least 34 passages), suggesting a strong link between this abnormality and the cell's adaptation to their new culture conditions[82]. In reality, a variety of genetic abnormalities could occur, during the differentiation process itself, at a significantly more rapid rate. In a study previously described, the authors analyzed the CNV occurrence during a 7 d experiment differentiating hESCs into motor neuron progenitors. They found an occurrence of partial duplication of 3 segments of chromosome 20 at day 7 in one of the differentiation experiments. This duplication was absent at day 2, suggesting that this type of abnormality could occur in a limited time, as short as 5 d[9]. Another article studied the presence of CNV in neuroprogenitors derived from a hESC line or a patient-specific iPSC line using CGH array. They demonstrated that these differentiated cells contained CNVs, including CNVs acquired from the PSC line (and detectable in this line) but also *de novo* CNVs generated during differentiation. Some CNVs were also shown to have been lost during the differentiation process, suggesting that maybe certain CNVs could offer a selective advantage or disadvantage for differentiation. On the other hand, Kammers *et al*[83] did not report any CNVs in iPSC-derived megakaryocytes compared to their undifferentiated counterpart, despite expected upregulation of highly biologically relevant gene such as those related to megakaryocyte development, platelet activation, blood coagulation *etc*.

Lastly, in the context of liver therapy, using three different hepatic differentiation experiments, we demonstrated that no *de novo* CNV were triggered using our differentiation protocol[33], but the time scale (22-24 d) of our differentiation protocol probably does not allow emergence of detectable CNV due to the limited number of mitosis. To our knowledge, there are no additional studies focusing on the impact of differentiation on iPSC genomic integrity. This could be partially explained by the fact that the majority of quality controls necessary for use of the cells in therapeutic applications are carried out at the iPSC stage, defining iPSC master cell banks, as it is commonly known how deleterious reprogramming can be on the cell genomic integrity. However, this option negates the direct impact of differentiation on the cell genome. Another reason for the lack of interest could also be due to risk minimization based on the fact that differentiated cells have a considerably decreased ability to proliferate, compared to their pluripotent counterparts (which should be eliminated upon differentiation or selectively removed during the process). However, in the case of liver cell therapy for example, in addition to transplanting fully mature and functional hepatocytes, an alternative could be to transplant hepatic progenitors which are able to mature *in vivo* in an optimal microenvironment. As these progenitors maintain their proliferation capacity, the concept would be to improve overall cell engraftment and proliferation once transplanted. Nonetheless, there is so far no consensus regarding the exact time point during the differentiation of the cells at which they will be optimum for use in transplantation. Moreover, large scale investigations using high-throughput genomic analysis techniques are yet to be carried out. Therefore it is imperative to continuously assess the impact of differentiation on the genomic integrity of cells, including the development of more reliable and efficient differentiation protocols which have the potential for use in clinics.

WHAT ARE THE IMPACTS OF GENOMIC ALTERATIONS IN HIPSCS AND THEIR DERIVATIVES?

Enforcing hiPSC genomic integrity quality control

The last section of the review will try to address the following question: What are the impacts of these genomic alterations in hiPSCs and their derivatives? This issue is not simple and has already been touched on in recent reviews[81,84]. For the purpose of discussion, let us assume that the relevance of the hiPSC mutations, and therefore the importance of performing genomic integrity quality control, is highly dependent on the application the cells will be used for. To illustrate this idea, various potential

examples of hiPSC based therapies will be discussed. In the first example, differentiated cells derived from hiPSCs may be used *ex vivo* to generate extra-corporal devices. More specifically, hiPSC-derived hepatocytes could be used in external bio-artificial devices aimed at temporarily replacing the liver for patients suffering from acute liver failure whilst they wait for an organ transplant. In this case, the important parameter is the functionality of the cells and because the cells are not injected into the patients, we could accept that mutations will not prevent their use in this application (assuming that such mutations do not have a direct impact on hepatic functions). Another example is the use of hiPSCs to perform personalized therapy to treat a children affected with a life threatening disease, as the strategy has long term benefits. Once the patient's cells are collected, patient's specific iPSC lines could be generated for the purpose of *in vivo* cell therapy. In this case, stringent genomic integrity controls should be performed in an attempt to identify a "safe" iPSC line (the questions of which exact tests need to be performed will be subsequently discussed). Ideally, autologous iPSCs would be the adequate strategy in order to avoid an immune response. However, despite the continual progress in the techniques of hiPSC reprogramming, culture and differentiation, it remains to be an expensive, long and arduous task. It appears that it is increasingly more feasible to use hiPSC banks with regards to providing large scale medical care (or semi-personalized hiPSC based therapy)[85]. For a successful organ or cell transplantation, there are three immunogenic challenges that need to be overcome; these include human leukocyte antigen (HLA), blood groups in some cases such as liver transplantation and minor antigens compatibility. For blood group compatibly, the selection of group O donors could avoid part of the immune reaction. However, HLA is one of the most polymorphic loci in the mammal's genome with thousands of alleles recognized. Various studies have estimated the number of pluripotent stem cell lines that are required to be stored in a national cell bank to cover the HLA diversity. Using the United Kingdom population as an example, 150 selected donors could match about 85% of the country population with minimal immunosuppression (including 18.5% with a full match)[86]. Similar prediction models have been calculated with hiPSCs and, according to one, a bank comprising of 100 hiPSC lines exhibiting the 20 most frequent HLA in each of the following populations would still exclude 22% of the European Americans, 37% of the Asians, 48% of the Hispanics, and 55% of the African Americans[87]; this highlights the fact that countries with more diverse populations would require higher number of cell lines to be stored within the cell bank. Indeed, Fraga *et al*[88] characterized 22 human embryonic cells and observed that only 0.011% of the Brazilian population could be matched with these cell lines. It should be noted that the Brazilian population is known for its high degree of genetic diversity. However, HLA compatibility would not negate the use of immunosuppressive drugs, as the role of minor histocompatibility antigens in the rejection process should not be underestimated when transplanting cells from genetically unrelated individuals[89]. In particular cases, depending on whether the cells are transplanted into immune privileged sites or for short periods of time, immune suppression may not be required. While generating such cell banks, which are likely to be feasible within an international consortium, parallel efforts should be carried out to screen the cells for genomic abnormalities prior to banking. Herein, the impact of cell culture and differentiation on the cell genome is neglected; as such an additional quality control step should be performed subsequent to the cells use in therapy. This is a supplementary control step and should not replace the baseline iPSC genomic integrity controls.

The next important question is to define which techniques or combination of techniques should be used to screen for genomic abnormalities. Some consensus and guidelines are slowly emerging but there is a lack of standardization worldwide. Despite that there is currently no defined consensus, some guidelines are slowly emerging[90]. Karyotype analysis is likely to be a requirement and could also be used to rapidly eliminate aberrant iPSC lines. CNVs should also be analyzed, using CGH array or SNP array[91]. However, only SNP arrays enable consecutive LOH regions detection attesting the possibility of UPD. Genome or exome sequencing is highly informative but specific care should be taken when using these techniques for CNV detection. Besides, Kang *et al*[92] highlighted the need to monitor mtDNA mutations in iPSCs, especially those generated from older patients, as well as metabolic status of those iPSCs. Finally, once the data is collected, the level of tolerance applied should be defined. It should be highlighted that the qualification will always depend on the resolution of the technique used and the analysis parameters: the stringency of the quality control required is directly linked to the functional impacts of these mutations and the application such cells will be used in.

The complexity of predicting the subsequent impact of genomic abnormalities

Assuming that the hiPSC lines available for banking will contain a few mutations, another challenge will be to assess whether these mutations could regard the cells as safe for use in clinics. Once again, the answer is not straight forward. For example, it has been estimated that about 12% of the human genome contains CNVs. These CNVs contribute to 0.12 % to 7.3% of the genome variability in humans[93] and are often benign. More than 300000 CNVs which are not associated with a clinical phenotype have been identified in the general population and are catalogued in the Database of Genomic Variants[94]. Several genes can be found within the boundaries of large CNVs and the resulting functional changes are not easy to predict. Genomic variants associated with clinical symptoms are shared through the DECIPHER interface[95]. Other online tools are able to help predict the effects of the variants such as Variant Effect Predictor[96]. These programs are examples of online tools used to help identify genomic abnormalities present in hiPSC lines and predict the possible effects. Despite the availability of such tools, each mutation should be individually considered, and its attributed importance will depend on the application the cells will be used for. Strong evidence exists on the potential impact of CNVs in the context of hiPSCs, described in a recent article which documents the generation of several integration-free hiPSC lines from patients affected with two neurodevelopmental disorders directly caused by CNVs of 7q11.23 locus[97]. The CNVs involve the loss or gain of approximately 28 genes, leading to Williams-Beuren Syndrom (OMIM 194050) in the case of deletion, or Williams-Beuren region duplication syndrome (OMIM 609757) in the case of duplication. Through hiPSC generation, the authors documented the CNV present in the patient's fibroblasts and notably performed transcriptional analyses of patient-specific iPSCs at the pluripotent stage and once they were differentiated into neuronal cells, cardiac cells and gastrointestinal cells. Compared to the control hiPSC lines, the study first showed that several hundreds of genes are differentially expressed; highlighting a network effect of the 7q11.23 dosage imbalance. They also observed that several of the affected pathways were already dysregulated at the pluripotent state, and various other expression changes were cell-type specific. This article confirms once more that hiPSCs can be good models to mimic pathologies and provides clear evidence of the functional impact of a pathologic CNV in hiPSCs and their derivatives. Importantly, showing that even at the undifferentiated state big abnormalities are patent, this example might indicate that previously undescribed mutations/CNV are innocuous when undifferentiated or differentiated hiPSCs bearing these abnormalities exhibit transcriptomes comparable to that of normal counterparts.

The link between pluripotency and tumorigenicity

The gene expression networks responsible for the pluripotency of hPSCs are closely related to those implicated in oncogenesis[98]; and the culture of hiPSC could be associated with the positive and negative selection of genes involved in oncogenesis or cell cycle regulation. Both pluripotency and oncogenicity are linked to high proliferation capacity, self-renewal and in some cases differentiation capacity. Key factors involved in the pluripotent network have been shown to be involved in the oncogenic processes. For example, the transcription factor NANOG plays a major role in the self-renewal of CD24+ cancer stem cells in hepatocellular carcinomas[99] and SOX2 promotes the survival of cancer stem cells in numerous malignant tumors including lung and esophagus cancers[100]. The use of integrative vectors for reprogramming is particularly dangerous in this context. One study showed that cMyc reactivation after reprogramming lead to the generation of tumors in chimeric mice[101]. Besides, a study described a long term (47 d) follow-up after the transplantation of hiPSC-derived neurospheres in a spinal cord injury mouse model. The authors described the occurrence of tumors linked with the reactivation of the Oct3/4 transgene associated with epithelial to mesenchymal transition based on transcriptomic analysis[102]. Another article highlights the direct and indirect interactions that exist between genomic integrity and pluripotency networks in human PSCs using transcriptomic and cistromic analyses[103]. Tumorigenicity is an intrinsic property of iPSCs and the advances of reprogramming, culture and differentiation protocols may not be sufficient to suppress the risk for use in clinical applications. With this regard, sorting-based strategies have been developed to purify the cell populations before transplantation. These strategies include cell selection using cytotoxic antibodies against PSCs[104], magnetic sorting depleting PSCs[105] or enrichment of the differentiated cells[106]. Estimations on the minimum number of cells sufficient to generate teratomas post injection of human PSCs in immunodeficient mouse have been investigated. Results suggest that 10000 cells are required if the injection is performed in the skeletal muscle and 100000 cells if injected into the myocardium[107]; this number being likely different if non-immunodeficient animals are used. In the case of the adult mouse liver in which 800000 hepatocytes should be

transplanted, 10000 cells represent only 1.25% of the entire population, signifying the importance of achieving a high differentiation yield and an effective cell sorting technique. In a Parkinson disease model in non-human primate, a study showed that the residual presence of ESCs in the preparation of neuronal cells differentiated from ESCs induced teratoma formation after cell injection in primate brains[108]. However, the injection of more mature terminally differentiated cells circumvented this outcome. Depending on the target organ, the maturity state of differentiated cells that is required has yet to be defined. However, satisfying transplantation results have been obtained in a non-human primate model using ESC-derived cardiomyocyte progenitors[109], suggesting progress within this area.

What are the alternatives?

This review highlights that iPSCs are prone to genomic instabilities and are intrinsically linked to tumorigenicity. Therefore the next question to address is whether we have access to another cell type equivalent to iPSC which could help circumvent this problem. Recent findings demonstrating that hPSCs could be derived by nuclear transfer into human oocytes (called NT-ESCs for Nuclear Transfer-Embryonic Stem Cells)[110] propose an alternative method of generating pluripotent stem cells with a desired genotype (albeit not avoiding ethical issues due to the need of oocytes). In this context, the genetic and epigenetic integrity of these cells have been compared with those of isogenic iPSCs (generated from the human fibroblasts used for nuclear transfer). In addition to showing similar gene expression and DNA methylation profiles, NT-ESCs and hiPSCs have comparable numbers of *de novo* coding mutations, suggesting that regardless of the derivation approach, the nuclear reprogramming itself is linked to genomic aberrations[111,112]. Another possibility to bypass this issue is to take advantage of the recent advances made in the field of trans-differentiation, also known as direct reprogramming. Human fibroblasts have been successfully transdifferentiated to hepatocytes[113], dopaminergic neurons[114] and cardiomyocytes[115]. However, it should be assessed whether these techniques are beneficial in terms of genomic integrity, as they avoid an important step of cell dedifferentiation. The epigenetic and genetic remodeling linked with trans-differentiation could be less detrimental when compared to reprogramming and the subsequent differentiation of the cells. Further investigation is required to fully understand the benefits of using novel strategies such as trans-differentiation to replace the use of iPSCs in cell therapy. However, it should be noted that trans-differentiation of somatic cells, which have a limited amplification capacity, will not solve the quantitative problem of cell availability.

CONCLUSION

Despite that hiPSCs are prone to genomic instability, a reliable quality control combined with optimized reprogramming, culture and differentiation conditions may be sufficient to minimize the impact on the cell genome. Nevertheless, a footprint-free cell population derived from iPSCs seems difficult to obtain for now and even the necessity to reach such a goal is questionable based on the planed application. Moreover, the fact that hiPSCs contain more genomic variations than cultured somatic cells is not obvious. A recent study derived subclones from fibroblasts and clonal iPSCs from the same population and highlighted by targeted deep sequencing that clonal iPSCs and fibroblast subclones displayed comparable numbers of *de novo* variants[21]. Thus, somatic mosaicism seems to be one important parameter, although underestimated because of the technical difficulties to detect mosaic below 5%-10% whatever the techniques used[116]. Finally, besides genetics, epigenetic factors have not been addressed in this review but may also play a role in the heterogeneity and behavior of hiPSCs and are important parameters to address in the quest of an optimal iPSC-derived population.

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REVIEW

Enhancing survival, engraftment, and osteogenic potential of mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs) are promising candidates for bone regeneration therapies due to their plasticity and easiness of sourcing. MSC-based treatments are generally considered a safe procedure, however, the long-term results obtained up to now are far from satisfactory. The main causes of these therapeutic limitations are inefficient homing, engraftment, and osteogenic differentiation. Many studies have proposed modifications to improve MSC engraftment and osteogenic differentiation of the transplanted cells. Several strategies are aimed to improve cell resistance to the hostile microenvironment found in the recipient tissue and increase cell survival after transplantation. These strategies could range from a simple modification of the culture conditions, known as cell-preconditioning, to the genetic modification of the cells to avoid cellular senescence. Many efforts have also been done in order to enhance the osteogenic potential of the transplanted cells and induce bone formation, mainly by the use of bioactive or biomimetic scaffolds, although alternative approaches will also be discussed. This review aims to summarize several of the most recent approaches, providing an up-to-date view of the main developments in MSC-based regenerative techniques.

Key words: Mesenchymal stem cells; Bone regeneration; Hypoxia; Anoikis; Preconditioning; Bioactive scaffolds; Senescence; Engraftment; Homing; Osteogenesis

Core tip: Mesenchymal stem cells (MSCs) are important tools for a wide range of

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therapeutic applications, including the treatment of critical size fractures or bone defects. However, whereas early clinical studies showed great expectations, long-term benefits of MSC-based treatments are not entirely successful. Transplanted cells had to face a series of important challenges that greatly reduce their survival and engraftment, and thus, their capacity to regenerate the target tissue. Although there is solid data indicating that the paracrine actions exerted by MSCs are equally important in the outcome of the treatment, this review is based on the current strategies aimed to enhance tissue regeneration directly occurring from the engraftment and differentiation of the transplanted MSCs.

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INTRODUCTION

First described by Friedenstein[1] in 1967, mesenchymal stem cells (MSCs) are adherent cells with a spindle shape, resembling fibroblasts, capable of self-renewal and differentiation into mesodermal lineages, such as osteocytes, adipocytes, or chondrocytes. This specific type of stem cells has unparalleled features that make them a unique and valuable tool for tissue repair and other cell-based therapies. In fact, nowadays, MSCs-based treatments are the experimental therapies drawing more attention in the generative medicine field, being the subject of nearly a thousand registered clinical trials, complete or on-going, worldwide (www.clinicaltrials.gov)[2]. Due to their ability to differentiate towards the osteogenic lineage, in the last few years, there has been an increasing interest in the use of MSCs-based approaches to improve bone repair and regeneration. In particular, the use of MSC-based therapies would certainly benefit the treatment of non-union fractures or critical size bone defects resulting from direct trauma or from the removal of large bone areas through surgical procedures in patients with osteosarcoma, necrosis, or other pathologies. Due to the known drawbacks of autologous and allogeneic bone grafts, bone tissue engineering has emerged as an interesting alternative and the combination of MSCs with biocompatible scaffolds represents a promising strategy for treating critical size or non-union fractures.

Unlike other stem cells, MSCs are able to maintain a high degree of plasticity, expressing also ectodermal and endodermal genes[3]. This gives them the ability of trans-differentiating and producing cells from other germ layers, thus challenging the previous concept that tissue-derived adult stem cells could only give rise to cells and cell lineages found in the tissue of residence. In fact, MSCs have been found to produce, under specific circumstances, skin, neural, and hepatic cells[4].

Although the regenerative potential of MSCs was initially linked almost exclusively to their ability to differentiate into multiple cell lineages once engrafted in the recipient tissue, nowadays the extensive paracrine activity seems to be the focus of many studies, since it appears to be directly related to the therapeutic action of MSCs. Besides being able to replace cells in damaged tissues, MSCs can also produce secretory factors that play critical roles in tissue repair and immune response modulation, and support both engraftment and trophic functions (autocrine and paracrine actions)[5]. Those secretory factors comprise cytokines, chemotactic factors, molecules involved in the remodelling of the extracellular matrix (ECM) and growth factors[6]. Thanks to the secretion of these molecules, after *in vitro* administration, MSCs can migrate to damaged tissue and promote the establishment of an anti-inflammatory environment that supports proliferation and avoids cell death, thus stimulating tissue remodelling and survival[7,8].

In addition to these properties, MSCs are generally easy to source from different adult tissues such as fat, blood, or dental pulp, using relatively simple, and minimally invasive procedures, making these cells very attractive for their use in the clinic. However, in relation to bone regeneration, MSC-based therapies, specifically bone marrow MSCs (BM-MSCs), which have associated a more complicated extraction method, seem to display the highest osteogenic potential when compared to MSCs sourced from other tissues. Adipose derived stem cells (ASCs) seem to have similar

osteogenic characteristics as BM-MSCs[9], but also possess the advantages of being easily isolated and of being present at a much higher concentration in the source tissue (500 times greater than that of the BM-MSCs)[10]. Although ASCs represent a good alternative to BM-MSCs due to these characteristics, the studies using these cells are still scarce and more information is needed referring to their usefulness in bone repair.

Despite having been proven to have short-term benefits, the long-term benefits of MSC-based therapies are not currently clear, and the final outcome of the treatments involving MSCs show high inter-patient variability[11]. Importantly, the limited benefits seen in clinical trials are linked to the low engraftment and survival rate of the transplanted MSCs, regardless of the tissue of origin[12], and to ineffective osteogenic differentiation. At this point, it is important to highlight that different characteristics of the transplanted MSCs are required depending on their subsequent application, that is, whereas homing would be crucial for the treatment of systemic bone loss, such as that linked to osteoporosis, this has no relevance when MSCs are used to build bone grafts *in vitro*.

Current studies in the field of tissue engineering are now focused on finding the appropriate conditions that would lead to successful tissue regeneration. One possible strategy to increase the success rate of the MSC-based techniques is producing cells that are able to resist the hostile microenvironment through what is called cell-preconditioning. This review will discuss the different approaches used for cell preconditioning, from the modification of culture conditions that promote cell survival and engraftment to the use of bioactive scaffolds that would increase the osteogenic capacity of the transplanted cells.

OPTIMIZING MSCs SURVIVAL AND ENGRAFTMENT

Cell survival, once transplanted in the recipient tissue, may be affected by length and culture conditions, such as the presence of serum or oxygen, mechanical stress during the implantation procedure, or cell death due to the lack of an anchorage among others. In the following sections, we will discuss the influence of all these factors on the success of the engraftment and the possible solutions proposed by different authors.

Optimizing in vitro culture conditions

Avoiding replicative senescence: The amount of MSCs that can be sourced from adult tissues is limited, thus, it is imperative to expand them *in vitro* in order to obtain the sufficient number of MSCs needed to achieve maximum therapeutic effect. However, clinical applications require that no differentiation potential is lost during the expansion process. This is particularly troublesome in the case of BM-MSCs, due to the low percentage of these cells present in the bone marrow, and therefore, the necessity of prolonged time in culture and increased passage number. This need for a high number of MSCs brings up one of the first limitations to their clinical use: their limited replicative lifespan. In fact, it has been estimated that MSCs cultured *in vitro* can achieve a maximum of 15 to 30 population doublings, depending on donor age[13,14]. Although this restricted proliferative capacity would represent a safety advantage, since it ensures a low probability of malignant transformation, a large scale *in vitro* expansion also leads to the loss of proliferation and differentiation capacity, which would deem them unsuitable for several regenerative procedures[15,16].

Telomere shortening, one of the main hallmarks of aging[17], has been measured during culture of MSCs. Various studies clearly demonstrate that telomere attrition leads to BM-MSC senescence[13] and in fact, this shortening has been even established on 17 base pairs lost on each MSC division *in vitro*[13,14]. However, other works claim that no changes in telomere length are detected after 25 passages[18], therefore, the relevance of telomere shortening in the acquisition of a senescent phenotype after prolonged *in vitro* culture is currently controversial. Another hallmark of aging[17], the accumulation of free radicals or reactive oxygen species (ROS), has been linked to a decrease in adhesion of MSCs[19], something crucial for the engraftment of the transplanted cells, and also to an increased adipogenic potential[20] that would hamper their use for bone regeneration techniques. Oxidative stress is also a factor directly linked to a decreased cell survival[21]. At this point, it is interesting to mention that pretreatment of MCSs with vitamin E, done by Bhatti *et al*[22], seems to result in a protective effect against oxidative stress by increasing cell anabolism.

During prolonged cell culture, MSCs also suffer changes that result in an inability to maintain the structure and function of chromatin, something indispensable for the correct execution of the gene transcription program[23,24]. Indeed, important changes in

DNA methylation have been detected during *in vitro* expansion of MSCs[25]. These and other changes at the level of the epigenome (*i.e.*, histone methylation or acetylation) would have an important impact on gene expression. In fact, according to Wagner *et al*[26], more than 1000 transcripts were up-regulated at least two-fold in senescent MSCs whereas over 500 transcripts were down-regulated. Part of these changes in gene expression levels would lead to the acquisition of a senescent state[27], the subsequent decrease in MSCs viability[21], and the loss of potential.

To prevent cultured MSCs undergoing replicative senescence during *in vitro* expansion, different approaches have been analysed. Some of those methods were based on preventing telomere shortening, due to the putative link between this process and MSC aging. One way to achieve this goal is to express the catalytic subunit of telomerase so that the cells can further divide without losing telomere length[28,29]. However, these modifications so far have been done using viral-based vectors as vehicles, and therefore this could potentially transform the recipient cells, precluding their use in the clinic.

More recently, a different approach, based on introducing variations in the culture media to avoid replicative senescence rather than on modifying the gene expression of the MSCs, has been tested. Grezella *et al*[30] tried to reduce the presence of senescent cells in MSCs cultures by using senolytic drugs. Although four different drugs were tested, only one of them (ABT-363_Navitoclax) seemed to have selectivity for senescent cells. The results, however, were not encouraging since this drug also affected non-senescent cells to some extent and no rejuvenation of MSCs was detected in terms of gene expression signature or telomere length. Other senolytic drugs are currently being investigated, which might, either on its own, or in combination with other compounds, have a clearer rejuvenating effect on MSCs in culture. Interestingly, other authors have managed to reduce the percentage of senescent MSC during *in vitro* expansion, by simply growing them in a defined xeno-free human plasma fraction[31] or in the presence of platelet lysate as a substitute of foetal bovine serum (FBS)[32]. This method has the additional advantages of avoiding the risk of transmission of zoonotic infections as well as immunological reaction to xenogenic supplements used in culture, such as FBS.

All in all, it seems clear that assaying MSCs aging "status" during their culture *in vitro* and positively selecting for non-senescent cells prior to their use in cell-based therapies would certainly improve the outcome of the procedures. This screening could be simply done by a variety of methods, with the easiest one being the observation of MSC size and morphology in culture, since MSCs rapidly loose their spindle shape and increase their size up to 10 times at later passages[26,33], a process associated with an increase in actin stress filaments[34]. This visual method, however, is highly subjective and does not allow accurate quantification of the percentage of senescent cells in culture. Bertolo *et al*[35] developed an *in vitro* expansion score to quantify the senescent state of MSCs and predict whether the cells would maintain their differentiation ability. By measuring population doubling time, senescence-associated β-galactosidase expression (SA-β-gal), cell size and telomere length, and assaying colony forming unit potential, these authors clearly demonstrated that whereas early passages of cells (from P1 to P3) maintained all their potential, at late passages (>P7) MSCs lost their osteogenic and chondrogenic potential while gained adipogenic potential. Another approach to increase the percentage of replicative active MSCs to increase chances of success in cell-based therapies would be to positively select cells free of senescence markers. Although scoring all the previous parameters will certainly help evaluate the state of MSCs, the various techniques involved made this process highly time-consuming. Interestingly, the same group recently published a fast and label-free flow cytometry-based approach to quantify the percentage of senescent cells in a given culture[36]. This method could be extremely useful to select MSCs with high regenerative abilities for subsequent applications.

Hypoxic preconditioning: Pathological conditions susceptible of being treated by MSCs transplantation are normally linked to the death of specialized cells in a particular tissue, as a result of toxic agents or autoimmune processes. The microenvironment surrounding this damaged tissue will have associated severe ischemic conditions (≤1% oxygen) that can also be the cause that triggered cell death. *In vitro* MSCs cultures are mainly maintained in normoxia (21% of oxygen), while the natural niche of the MSCs has a constant moderate hypoxia with concentrations ranging from 1% to 7% oxygen[37]. This restricted hypoxic microenvironment found in diverse pathological tissues also applies to bone defects, where the hypoxic conditions (<1% oxygen) close to the anoxia that can be found in the fracture microenvironment following bony injury, are favoured by the low vascularization at the implantation site.

The dramatic transition suffered by the transplanted cells going from normoxia to

hypoxia or anoxia, could be alleviated by cell preconditioning[38,39]. Since transplanted MSCs are likely to be placed in a hypoxic environment, culturing the cells in hypoxic conditions might improve their survival. Besides reducing the percentage of oxygen in the culture settings, addition of other drugs might help the engraftment process. In fact, Zhang *et al*[40] showed that BM-MSCs that have been preconditioned in hypoxia (0.1% oxygen) in serum free medium and in the presence of 0.5 mmol/L dimethyloxaloylglycine (DMOG) had an improved angiogenic capacity. This improvement was related to the upregulation of hypoxia inducible factor-1α (Hif-1α), which enables cells to survive in oxygen deprivation conditions by providing oxygen-independent adenosine triphosphate (ATP) production or by inhibiting apoptosis induced by hypoxia. Importantly, after being cultured in those conditions, MSCs also showed a greater osteogenic and regenerative potential even in aged animals, where the MSCs potency is known to be already very limited[40-42].

Other pharmacological agents are also able to improve cell survival when MSCs face a hypoxic microenvironment. Pretreatment of MSCs with trimetazidine enhanced cell viability when cells are re-oxygenated after being exposed to hypoxic conditions. The effect of trimetazidine might also be mediated by HIF-1α *via* upregulation of the anti-apoptotic gene Bcl-2, and downregulation of Bax, an apoptotic gene[43]. Kheirandish *et al*[44] developed a system of preconditioning consisting of culturing the cells during 15 min in 2.5% O_2, re-oxygenation for 30 min in 21% O_2, and hypoxia preconditioning in 2.5% O_2 during 72 h. This system seems to significantly improve the proliferation and migration abilities of MSCs *in vitro*. According to these authors, the re-oxygenation after a few minutes of hypoxia improves the expression of pro-survival genes as well as the expression of various trophic factors, angiogenic factors, VEGF, and basic fibroblasts growth factor (bFGF) in MSCs[45,46]. Moreover, this re-oxygenation process also results in a decrease of caspase-3/7 activity and lactate dehydrogenase release, decreasing the sensitivity of the cells to the ischemic microenvironment[47,48]. Another important point in favor of MSC preconditioning in hypoxic conditions is the evidence that this procedure seems to inhibit the malignant transformation of MSCs after transplantation[49].

Regarding specifically to the effect of hypoxic preconditioning in bone regeneration, in animal models, hypoxic conditioning seems to lead to an enhanced angiogenic and osteogenic potential[50,51]. Also, in human MSCs, there are data indicating that culturing MSCs in 2% and 5% O_2 highly favors their proliferation and increases their osteogenic differentiation[52,53]. In addition, one of the factors that reduces cell survival when MSCs reach the target tissue is oxidative stress[21]. Interestingly, pretreatment of MCSs with vitamin E, as described by Bhatti *et al*[22], results in a protective effect against oxidative stress by increasing anabolism of the cells.

It is important to highlight that when MSCs are re-implanted, they not only have to face hypoxia to the point that they can become apoptotic, but they also have to face a lack of nutrients[54,55]. Wang *et al*[54] observed that MSCs preconditioning with a low dose of lipopolysaccharides reduced the apoptosis induced by hypoxia and nutrient depravation by inhibiting the downregulation of CX43, a process apparently related with the Erk signaling pathway. Sun *et al*[56] demonstrated that preconditioning of MSCs with sevoflurane not only minimized cell apoptosis when exposed to hypoxic-serum deprived media but also enhanced MSC migration, suggesting that this improvement in the therapeutic potential of MSCs might be related to the upregulation of HIF-1α, HIF-2α, VEGF, and pAkt/Akt.

Three-dimensional (3D) cultures: 3D cultures of MSCs, called spheroids, have been shown to increase the expression of homing-related genes[57], angiogenic and growth factors[58,59], and anti-inflammatory and immune-modulator compounds[60-63]. Besides, 3D cultures also improve cell survival, promoting the expression of anti-apoptotic genes and inhibiting the expression of pro-apoptotic genes[60,64]. MSCs cultured in spheroids present higher expression of pluripotency-related genes, leading to an increased potency and trans-differentiating capacity[65,66]. Importantly, MSCs obtained from spheroids present a smaller size, which may improve intra-venal administration by avoiding lung-trapping[60], something to take into account if intravascular delivery is involved in the procedure. Although these enhanced capabilities are related to 3D culture, their acquisition also depends on the culture conditions of the spheroids[60,61,64]. Despite the fact that the concentration of oxygen in core of spheroids is reduced, Murphy *et al*[67] observed that changes in MSCs expression pattern are not oxygen mediated, which might induce to think that the improvement associated to this culture method should be due cell-to-cell interactions. Regarding the effect of spheroids in bone regeneration, Ma *et al*[68] observed a significant improvement in bone formation after implantation of MSCs spheroids, with a high rate of survival and retention at the injection site in murine models. It is important to note that the bone

tissue formed from MSCs spheroids presents similar histological characteristics to native bone, as well as a good mechanical strength[69].

Administration and implantation procedure

Selecting the appropriate administration route for MSCs delivery: In the field of MSC-based bone regeneration, different delivery approaches have been tested for the transfer of MSCs to the site of damage. There are mainly three different ways of administering MSCs: local injection directly to the site of damage, systemic injection, and *via* biocompatible scaffolds.

Use of local or systemic injection for MSC delivery: There is mounting evidence indicating that both the correct route of administration and the proper dose can increase the success rate of MSCs therapies[70,71]. More clinical studies are necessary to determine these two parameters in order to achieve the maximum therapeutic effect in different diseases.

Systemic administration of MSCs has been widely studied. Intra-vascular delivery of MSCs is the less invasive route for MSC delivery and thus, the more interesting from the clinical point of view. Although this route has shown some benefits avoiding intervertebral disc degeneration in a murine model,[72]intra-venous delivery of MSCs has important drawbacks. The main downside of this delivery route is the fact that a high percentage of the administered cells could be entrapped in the lungs, something known as pulmonary first-pass effect[73], and in other organs such as the liver[74], forming microemboli that can have severe consequences to the functionality of those organs. Intra-arterial administration was considered a good alternative to avoid MSCs retention in the lungs and increase the homing rate of the cells[75,76]. However, despite this apparent benefit, Cui *et al*[75] also detected the formation of micro-occlusions in a cell dose-dependent manner in murine models, bringing important safety concerns to the use of the intra-arterial route. In animal models, systemic administration to treat generalized bone loss associated with osteoporosis has been tested with unclear results. Whereas one study demonstrated that systemic administration of allogenic MSCs had no obvious effect on osteoporotic bone loss in ovariectomized rats, another group reported that repeated injection of allogeneic MSCs might promote fracture healing when combined with local administration[77]. However, a few studies have shown the usefulness of MSC intra-arterial administration of MSCs in humans. Direct injection of BM-MSCs into the defect is widely used to treat non-union fractures with a high percentage of patients achieving union one year after the treatment[78]. Treatments of steroid-induced osteonecrosis using MSCs delivered *via* the medial circumflex femoral artery have also proven satisfactory after 5 years[79]. An alternative route for administering MSCs in osteonecrosis treatment is the administration with core decompression[80]. This has also given good results although it has normally been performed with bone marrow concentrate and not with *in vitro* expanded autologous MSCs[81].

Intramuscular administration of MSCs has been recently suggested as a better alternative to intravenous administration[82]. Different MSC administration routes, including intravenous, intraperitoneal, and subcutaneous, were compared to intramuscular administration. Whereas intravenously infused MSCs were not detectable just a few days after administration, and intraperitoneally and subcutaneously delivered cells were detected up to 3-4 weeks, intramuscularly delivered MSCs achieved more than 5 months of survival *in situ*. In spite of these results, it is still not clear whether this administration route can be effective to treat bone related diseases or other kind of pathologies.

Use of biocompatible scaffolds for MSCs delivery: In an attempt to increase the retention rate of MSCs, cells can also be applied in association with certain biocompatible scaffolds. The use of scaffolds responds to the increasing evidence indicating that MSCs prefer 3D culture conditions and that, after seeding, cells are able to survive in the scaffolds, probably because these conditions are closer to their natural environment than the monolayer two dimensional (2D) culture[83]. When cells reach a high confluence after culture in monolayer, it is necessary to detach them from the dish, which leads to a down-regulation of important cell adhesion genes[84], and the subsequent decrease in engraftment efficiency after cell infusion. Thus, culturing the cells onto a support or scaffold may improve the engraftment of cells and the result of certain cell-based therapies.

Plenty of different scaffolds have been designed to improve different parameters such as cell survival, proliferation, and differentiation. Although initially scaffolds were mainly based on the use of hydroxyapatite and tricalcium phosphate, the latest generation of scaffolds trying to resemble the properties of bone microenvironment are based on natural polymers, such as alginate, collagen, chitosan, or cellulose, which

are subjected to a biomimetic mineralization process[85]. Of those, collagen is probably one of the main materials used because of the high biodegradability and biocompatibility, although it is commonly used in combination with other biomaterials, such as ceramic nanofibers[86,87]. As we will discuss later, it is also important to highlight that scaffolds could be modified to create bioactive structures that favour engraftment or elicit appropriate responses for specific applications, such as promoting bone formation.

Bioprinting has also emerged as an alternative for artificial bone generation. This technique aims to produce a construct with a pre-defined 3D architecture resembling the original tissue. Bioprinting holds a great potential for producing tissues in a patient-specific manner[88]. The development of biocompatible inks that can undergo a transformation from a liquid to a gel-like structure is crucial for the success of bone bioprinting. In this sense, hydrogel based bioinks seem to be a good choice[89,90]. Despite the promising future of bioprinting, this is a rather new technique and more work needs to be done to overcome some challenges and limitations of the current techniques besides the choice of bioink, such as the optimal cell source or the best bioprinting method for replicating heterogeneous tissues and organs[91].

Another approach uses what is called ECM powder to improve biocompatibility of different materials[92]. Cells can be directly cultured over the powder and injected into the patient or alternatively, ECM powder can be used to coat biomaterials with poor biocompatibility, improving cell engraftment after transplantation[92]. Interestingly, an osteoblast-derived ECM has proven to stimulate osteogenesis and promote bone formation[93,94]. Mao *et al*[95] cultured fibroblasts, chondrocytes, and osteoblast over microfibers of tyrosine-derived polycarbonates (pDTEC) until the cells released ECM over all the scaffold surface and then decellularized the scaffolds to preserve the ECM. This pDTEC-ECM showed enhanced chondrogenic and osteogenic differentiation[95]. Some authors have proposed that the osteoblast-derived ECM could be used to coat titanium scaffolds[96], which have shown unparalell mechanical properties and are routinely used in bone tissue engineering for orthopaedic implants in load-bearing areas. Despite these positive aspects, the difficult production of the ECM-powder currently hinders its application[92]

Avoiding mechanical stress during administration: As previously stated, administration of cells by injection has the advantage of being less invasive and therefore it is gaining popularity for clinical applications. However, during the procedure of injection, if cells are resuspended in low viscosity solutions, the mechanical stress can cause cell membrane disruption and subsequent cell death in a high percentage of the population[97]. This importantly limits the successful use of MSCs injection for regenerative approaches. In order to optimize delivery protocols and avoid mechanical stress during MSCs injection, cells could be suspended in a hydrogel that will encapsulate and protect them from membrane disruption. Although these hydrogels can lose viscosity due to stress in the syringe, they can still protect, to some extent, the cells from mechanical stress, slightly improving the survival rate. The most common hydrogels used for this procedure are alginate hydrogels[97], hyaluronic acid-based hydrogels[98-100], supramolecular beta-hairpin hydrogels[101], and protein-assembled hydrogel[102,103]. However, the microenvironment provided by the hydrogel implies a limited interfacial interaction between the cells and the hydrogel material, allowing only weak dynamic interactions between them, such as hydrogen bonds or hydrophobic and electrostatic interactions. These weak associations are lost during injection when the hydrogel is exposed to shear-stress, leading to restricted tissue regeneration. In order to improve this delivery method, Zhao *et al*[104] designed a strategy based on the use of microfluidics-assisted technology to encapsulate bone marrow-derived MSCs (BMSCs) and growth factors in photo-crosslinkable gelatin methacryloyl (GelMA) microspheres. This type of encapsulations, known as microcarriers, offers mechanical stress protection and also allows a high-cell density administration, which improves cell secretion of paracrine factors and enhances cell differentiation, improving their therapeutic effect [105]. Also, using four different microcarriers (Cytodex, Cytodex3, SphereCol, and Clutispher-S) and a reduced number of cells, Lin *et al*[106] were able to demonstrate improved cell proliferation and also better chondrogenic differentiation. Alginate microcarriers covered with silk are also a good alternative, as they can be used to culture MSCs *in vitro* with good rates of cell adhesion, proliferation, and differentiation, reducing cell manipulation, as these microcarriers can be directly transplanted into the patient[107].

Promoting cell homing: According to Karp *et al*[108], MSC homing can be defined as "the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium". We have already discussed how systemic infusion of MSCs is an interesting approach because of the minimally invasive procedure associated. An

important barrier for achieving successful regeneration of the target tissue when this delivery method is used, is the inability of targeting the exogenously infused MSCs to the tissues of interest with a high efficiency, that is, the inability of the cells to accomplish homing.

Despite the fact that the exact mechanism of MSC homing to the injury site is not completely elucidated, we know that homing is a multistep process, where chemotactic factors released at the site of damage play an essential role[109]. Chemoattraction of MSCs into the target tissue appears to be mainly mediated by the stromal derived factor (SDF-1)/CXCR4 axis[110-112], but in MSCs migration there might also be a contribution from monocyte chemoattractant protein/CCR2 and the hepatocyte growth factor/c-met signaling pathways, and from cytokines such as TGF-β1, IL-1β, TNF-α, or G-CSF[109,110,113]. Circulating MSCs are attracted by the SDF-1 secreted by the injured tissue; subsequently, interaction of MSCs with the endothelial cells through the P- and E-selectins leads to MSCs rolling over the endothelium. Afterwards the attachment of MSCs is mediated not only by SDF-1 but also by ligands, such as VCAM-1 and ICAM-1, β1 integrin, and very late antigens-4[109]. During transmigration, MSCs also need to cross the basement membranes located between the endothelial cells and the targeted tissue. In this step, matrix metalloproteinases (MMPs) have a crucial role. The secretion of MMP-2 and MMP-9, the main MMPs involved in MSC migration, is stimulated by the CXCR4 receptor activation and also by inflammatory cytokines such as TGF-β1, IL-1β, and TNF-α. MMP-2 is released as a pro-enzyme, proMMP-2, which will be activated by the tissue inhibitor of metalloproteinases-2 (TIMP-2) and membrane type1 (MT1)-MMP, also released constitutively by MSCs[113].

To improve MSC homing, it is possible to perform a preconditioning during cell culture. In fact, Kim *et al*[114] observed that pretreatment of MSCs with a glycogen synthase kinase-3 inhibitor increased cell migration after transfusion by promoting cell expression of CXCR4. The presence of HIF-1α also improves MSC migration *via* a similar mechanism, leading to the enhancement of CXCR4 and CCR2 expression, which recognizes damaged-tissue signals. In this case, there is also an enhancement of proteolytic enzymes, such as MMP-2 and MMP-9, necessary for the cells to reach the damaged region by degrading ECM[115]. Cell migration can also be promoted by enhancing the expression of MMP2 through the exposition of cells to erythropoietin and G-CSF, as confirmed by Yu et *al*[116]. Other strategies of tissue pretreatment have been tested. Zhang *et al*[86] saw increased capillary permeability and expression of VCAM-1 in the renal interstitial after ultrasound-targeted microbubble destruction (UTMD), improving MSCs migration and retention in the kidney. Li *et al*[117] also noticed an increase in SDF-1 and CXCR4 expression after UTMD as intravenous infusion of MSCs was performed in ischemic myocardium, which led to a higher retention of MSCs in the tissue. Najafi *et al*[115] demonstrated that pharmacological pretreatment with deferoxamine leads to an accumulation of HIF-1α in the cells. Liu *et al*[118] noticed that pretreatment of MSCs with SDF-1, secreted by the injured tissue, activates the signaling pathways Akt and Erk, leading to an increased ratio of Bcl-2/Bax with pro-survival consequences, concluding that through CRCX4 receptor, pretreatment with SDF-1 increase MSC migration, survival, proliferation, and secretions. Despite the fact that some studies observed that cytokines such as IL-1β impair bone formation by inhibiting MSC proliferation, migration, and differentiation[119], Carrero *et al*[120] described an increase in cell migration and adhesion due to the secretion of chemokines and growth factors induced by pretreatment with IL-1β. Other tissue pretreatments were also observed to enhance MSCs migration and homing. Hepatic radiation prior to MSCs transplantation ameliorates hepatic fibrosis in an animal model[121] and extracorporeal shock wave positively modifies the microenvironment to favor MSC homing for spinal cord injury[122].

Avoiding anoikis: All mammalian cells forming part of a tissue are surrounded by an ECM, which function goes far beyond offering structural support. The ECM also provides biochemical and biomechanical signals that have an important role in cell function regulation. Anoikis (greek word for homelessness) is the name given to the induction of cell apoptosis that occurs in anchorage-dependent cells in response to inappropriate interaction between the cell and the ECM[123,124]. Together with the harsh environment found on the recipient tissue, anoikis is one of the important barriers to a successful engraftment. It is important to note that using microarray and proteomic screening, Copland *et al*[125] identified plasminogen activator inhibitor-1 (PAI-1), a protein that inhibits cell migration as up-regulated in mouse and human MSCs under hypoxic conditions. The MSCs isolated from PAI-1 knockout mice showed more survival and adhesiveness than wild-type MSCs after transplantation on Matrigel. These findings corroborate that PAI-1 negatively regulates transplanted MSC survival and adhesiveness *via* promoting anoikis[125], establishing a link between this process

and hypoxia.

Anoikis is highly important when the cells are delivered through local or systemic injection. To promote cell survival in these conditions, integrins need to be bound to immobilized ligands. An approach that has already been discussed in the previous section is to mimic the adhesive response of these cells in suspension by encapsulating the cells in a provisional hydrogel matrix[126] that will preserve cell adhesion in what can be called a portable microenvironment, while the cells are travelling through the vasculature until they can engraft in the recipient tissue.

An interesting report by García *et al*[127], used protease-degradable polyethylene glycol hydrogels functionalized with an $\alpha_2\beta_1$ integrin-specific peptide (GFOGER) or an $\alpha_v\beta_3$ integrin-targeting peptide and loaded with VEGF to study whether the integrin-specific biomaterials modulate the effect of VEGF on bone regeneration. These authors demonstrated that both types of scaffolds have different effects when applied to critical size segmental defects in a murine model, highlighting the importance of integrin specificity in engineering constructs for vascularization and associated bone regeneration.

Another valid approach to improve endothelial adhesion, avoid anoikis, and thus enhance the survival of transplanted cells, would be regulating the levels of integrins and conexins, the main molecules involved in cell adhesion to the ECM. In agreement to this, MSCs homing is enhanced, *via* the SDF-1 axis[128,129], by the transfection of these cells with a vector expressing CXCR4. Also, ectopic expression of $\alpha_1\beta_1$ integrin in MSCs ameliorates homing to the bone in mice. The aforementioned approaches enhance gene expression by using viral vectors, which ensures long-term expression of the transgene, but clearly precludes their use in clinical practise.

ENHANCING OSTEOGENIC DIFFERENTIATION

Since MSCs have the potency to produce different cell types, a successful bone tissue engineering technique requires a way of preferentially inducing bone formation over the formation of other possible tissues. The osteogenic and adipogenic differentiation of MSCs are carefully balanced and, more important, mutually exclusive processes. This is highlighted by the fact that inhibition of adipogenesis seems to improve bone development and repair[130,131].

Although it is possible to genetically engineer the MSCs to promote cell differentiation, the viral nature of the vectors normally used to achieve this end could lead to unregulated cell growth and a markedly increased risk of tumour formation. An alternative approach to promote osteogenic differentiation consists in modifying the microenvironment surrounding the cell.

Many treatments have tried to induce osteogenesis while preventing adipogenesis to improve cell therapy for bone regeneration. Luo *et al*[132] observed that in canine MSCs, pharmacological pretreatment with icariside II may promote osteogenic differentiation *via* PI3K/Akt/mTOR/S6K1 signaling pathways. Wan *et al*[133] noticed that preconditioning of MSCs with rapamycin promoted cell osteogenesis by activation of autophagy. On the other hand, Lu *et al*[134] demonstrated an improvement of ASCs mobilization, proliferation, and osteogenic differentiation after pretreatment with TNF-α. Bisphosphonates are commonly used as treatments against bone diseases related to exacerbated bone resorption, such as osteoporosis. Hu *et al*[135] observed an interesting dose-dependent effect in MSCs pretreated with zoledronic acid (ZA), a commercial bisphosphonate. When MSCs were exposed to high doses of ZA, there was an inhibition of cell proliferation, while low dose pretreatments would induce the upregulation of osteogenic-related genes, such as alkaline phosphatase (*Alp*), osterix (*Osx*), and bone sialoprotein (*Bsp1*), which translated into an induction of osteogenic differentiation. Although TGF-β was initially described to inhibit both osteogenic and adipogenic differentiation, Van Zoelen *et al*[136] described an inhibitory effect of this cytokine over adipogenic differentiation of MSCs, and an enhancement of osteogenic differentiation. Some pathologies linked to bone lose are characterized by low magnesium concentration[137]. In relation to this, magnesium supplementation has been shown to improve osteogenesis and tissue mineralization in a dose-dependent manner, although the mechanisms involved in this process are not well understood[138].

Bioactive scaffolds represent a valuable alternative to provide molecular cues for the seeded cells and not only physical support. These molecular cues can drive the activation of intracellular signalling pathways promoting osteogenic differentiation[139]. The most common way to currently promote MSC osteogenic regeneration in the clinic is the concomitant administration of bone morphogenetic proteins (BMPs). Binding of a BMP homo- or hetero-dimer to a BMP receptor,

activates intracellular downstream Smad proteins that translocate into the nucleus, where they interact with *Runx2*, the master osteogenic regulator, to activate the expression of osteogenic genes[140]. One recurring problem observed in the regenerative treatments that are accompanied by the administration of BMPs, is the presence of important dose-dependent side effects. One possible solution is the use of scaffolds that locally and sustainably release low dose BMPs, alone or in combination with other osteogenic factors, such as TGF-β-1 o MMP10[141,142], to the bone defect microenvironment. Such scaffolds have proven to be very successful in animal models. Although these bioactive scaffolds were mainly alginate based, it has also been possible to increase bone formation by a dual delivery of MSCs and BMP-2 in a coral scaffold as Decambron *et al*[143] were able to observe.

Although silk fibroin-chitosan (SF-CS) scaffolds are able to improve cell adhesion without the need of adding any molecules, Tong *et al*[144] were able to increase the benefits of this scaffolds by adding TGF-β1, leading to enhanced proliferation and osteogenic potential in an animal model. Scaffolds made of poly(L-lactic acid) nanofibers have been seen to be a good support for MSC proliferation and osteogenic differentiation[145], but it is possible to improve these properties by coating these scaffolds with baghdadite[146], nanobioactive glass[145], nanohydrohyapatite[147], or willemite[148].

Lee *et al*[149] developed a bio-ink consisting in a hybrid hydrogel with a base of hyaluronic acid complemented with different peptides. Their objective was to improve angiogenesis and osteogenesis, two processes that are crucial in bone regeneration. With this in mind, bio-ink complemented with (1) substance P (SP) was able to promote not only angiogenesis but also enhanced expression of osteogenic genes such as RUNX2 and ALP; or (2) BMP-7D led to high osteogenic differentiation with also an angiogenic effect over MSCs.

As hypoxic stress is an important factor to decrease cell engraftment, Alemdar *et al*[150] developed a calcium peroxide (CPO) laden GelMA able to produce oxygen under hypoxic conditions during 5 days, reducing the possible tissue necrosis that can appear due to the hypoxic conditions. Another important fact reducing cell survival once in injured tissue is the oxidative stress due to high amount of ROS. To avoid this, Dollinger *et al*[151] developed a triblock polymer with protective properties against ROS.

CONCLUSION

Bone microenvironment shows high complexity in terms of composition and geometry and therefore, designing a scaffold that can imitate those particular conditions is a challenging process. A relatively recent advance in tissue engineering involves the use of biomimetic scaffolds. These scaffolds do not need any morphogens or biomolecules to activate osteogenesis. Instead, they have a matrix that per se initiates bone formation[152]. It seems that scaffold geometry has a critical role in initiating bone formation and this process is known as geometric induction of bone formation. Cells are able to sense surface roughness through differences in focal adhesion that are translated into morphological changes[153]. It has been proposed that this feeling of the geometry of the scaffold occurs through actin-myosin contractions[153-155]. This is indeed an interesting approach to eliminate all side effects that could rise as a consequence of the presence of different molecules or factors.

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REVIEW

Effect of poly(3-hydroxyalkanoates) as natural polymers on mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs) are stromal multipotent stem cells that can differentiate into multiple cell types, including fibroblasts, osteoblasts, chondrocytes, adipocytes, and myoblasts, thus allowing them to contribute to the regeneration of various tissues, especially bone tissue. MSCs are now considered one of the most promising cell types in the field of tissue engineering. Traditional petri dish-based culture of MSCs generate heterogeneity, which leads to inconsistent efficacy of MSC applications. Biodegradable and biocompatible polymers, poly(3-hydroxyalkanoates) (PHAs), are actively used for the manufacture of scaffolds that serve as carriers for MSC growth. The growth and differentiation of MSCs grown on PHA scaffolds depend on the physicochemical properties of the polymers, the 3D and surface microstructure of the scaffolds, and the biological activity of PHAs, which was discovered in a series of investigations. The mechanisms of the biological activity of PHAs in relation to MSCs remain insufficiently studied. We suggest that this effect on MSCs could be associated with the natural properties of bacteria-derived PHAs, especially the most widespread representative poly(3-hydroxybutyrate) (PHB). This biopolymer is present in the bacteria of mammalian microbiota, whereas endogenous poly(3-hydroxybutyrate) is found in mammalian tissues. The possible association of PHA effects on MSCs with various biological functions of poly(3-hydroxybutyrate) in bacteria and eukaryotes, including in humans, is discussed in this paper.

Key words: Mesenchymal stem cells; Polyhydroxyalkanoates; Poly(3-hydroxybutyrate); Tissue engineering; Differentiation; Natural functions; Microbiota; Bone; Intestine

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Core tip: Biodegradable and biocompatible polymers, poly(3-hydroxyalkanoates) (PHAs), are actively used for the manufacture of scaffolds that serve as carriers for mesenchymal stem cell growth in tissue engineering. It was shown that PHAs have their own biological activity affecting mesenchymal stem cell growth and differentiation. However, the mechanisms of the biological activity of PHAs remain unclear. In this review, we discuss the possible association of the effects of bacteria-originating PHAs on mesenchymal stem cells with various biological functions of PHAs in bacteria and eukaryotes, including in humans.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent progenitor stem cells that can differentiate into multiple mesenchyme cell types, including fibroblasts, osteoblasts, chondrocytes, adipocytes, and myoblasts, and this capacity contributes to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma. Due to these properties, MSCs are now considered one of the most promising cell types in the field of tissue engineering[1,2].

Tissue engineering is a multidisciplinary scientific, technological direction that combines the latest developments in engineering, materials science, cellular biology, biochemistry, and medicine, thus offering new approaches for the restoration of tissue functions. Tissue engineering is a fundamentally different paradigm in relation to surgery and transplantation. It is based not on approaches related to the substitution or functional compensation of tissue but on tissue regeneration: the organism itself can repair the damaged tissue if the appropriate conditions are provided. Tissue engineering involves an appropriate combination of cells (*e.g.*, MSCs), scaffolds, and bioactive molecules. Together, these interactions allow intercellular communication and cell-biomaterial interactions, which work toward achieving the desired therapeutic response[3,4].

Different types of biomaterials can be used for the development of scaffolds for tissue engineering: metals, ceramics, and synthetic or natural polymers have been used. Nevertheless, biodegradable and biocompatible polymers of natural or synthetic origin, polyhydroxyalkanoates, are believed to be one of the most promising biomaterials for developing scaffolds for bone regeneration. Since the beginning of the 21st century, medical devices and dosage forms based on polyhydroxyalkanoates have been actively introduced into medical practices. The following types of chemically synthetic polyhydroxyalkanoates are actively used in clinical practice and scientific research: poly(2-hydroxypropanoic) (polylactic, PLA, polylactides), poly(2-hydroxyacetic) (polyglycolic, PGA, polyglycolides), poly(6-hydroxycaprolactone), and their copolymers, for example, polylactide-co-glycolides (PLGAs) and polymers similar in structure, such as poly-p-dioxanone[5]. Natural polyhydroxyalkanoates – poly(3-hydroxyalkanoates) (PHAs) represent polyesters of 3-hydroxyalkanoic acids, *e.g.*, poly(3-hydroxybutyrate) (PHB) is a linear polyester of 3-hydroxybutyric acid. The biopolymer includes only the R-form of 3-hydroxybutyric acid due to which it is a partially crystalline polyester in an isolated and purified form (the PHB crystallinity is 55%-80%). For industry and research purposes, natural PHAs can be produced biotechnologically by bacterial producer strains[6]. The following natural PHAs can be distinguished depending on the length of the alkane side radical: PHB, poly(3-hydroxyvalerate), poly(3-hydroxyhexanoate), poly(3-hydroxyoctanoate), *etc*. During bacterial biosynthesis, as a rule, it is not homopolymers of other PHAs but their block-copolymers with 3-hydroxybutyrate that are the most frequently obtained: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), poly(3-hydroxybutyrate-co-3-hydroxyoctanoate), *etc*. All of them are quite different in their physicochemical properties, such as crystallinity, melting temperature, glass transition temperature, hydrophobicity, plasticity, elastic modulus, and others[7,8].

The chemical structure and the main properties of synthetic poly(2-hydroxyalkanoates) (PLA, PGA, and their copolymers PLGA) are similar to the

chemical structure and the physicochemical and biological properties of natural PHAs: the ability to biodegrade in an organism without the formation of toxic products; biocompatibility with human organs and tissues; optimal mechanical properties (relatively high strength, plasticity); other physicochemical properties (thermoplasticity, specific diffusion properties); and the opportunity to use efficient technological processes for their production[5-9]. Therefore, the study of natural PHAs (including PHB) as biomaterials for the regeneration of different tissues and organs is of paramount importance. Chemically synthetic PHAs can be considered biomimetic analogs of PHB[5,9]. However, it should be taken into account that the properties of natural biopolymers are determined by their functions in nature[10]. In particular, the properties of bacteria-origin biopolymers are closely associated with their role in the bacterial world. In most cases, this important information is not taken into account in research on the biomedical applications of various biomaterials. However, why should we not give it proper importance? In this review, we attempt to fill these gaps in the current scientific field by considering the natural properties of polymers in connection with their effect on cells.

A high *in vitro* biocompatibility of PHB has also been shown in studies using cell cultures. Therefore, PHB is a promising material for tissue engineering. Cell cultures of various origins, including murine and human fibroblasts, rat, rabbit, and human MSCs, human osteogenic sarcoma cells, human epithelial cells, human endothelial cells, rabbit articular cartilage chondrocytes, rabbit smooth muscle cells, and human neurons in direct contact with PHB when cultured on polymer films and scaffolds exhibited satisfactory levels of cell adhesion, viability, and proliferation. However, the study of the effect of PHAs on the growth and differentiation of MSCs is of particular importance for the use of PHAs as biomaterials for tissue engineering[8,11].

OVERVIEW OF THE EFFECT OF PHAS ON MSC GROWTH AND DIFFERENTIATION

Osteogenic differentiation of MSCs in vitro

It was shown that human[12,13], rat[14-17], and rabbit[18] MSCs isolated from both adipose tissue[14,15] and bone marrow[14-17], murine calvarial preosteoblast cells MC3T3-E1 S14[19], and human induced pluripotent stem cells[20] grown on scaffolds from PHB and its copolymers PHBV and PHBHHx undergo spontaneous differentiation in the osteogenic direction in regular medium. Osteogenic differentiation in osteogenic medium is more pronounced (Table 1). The osteogenic differentiation of MSCs and osteoblastic cells grown on PHA scaffolds was confirmed by a change in cell morphology[13,15,17,18,21], inhibition of their proliferation[14,15,17], an increase in alkaline phosphatase (ALP) activity, deposition of calcium salts[12,15,22], and the expression of markers of osteogenic differentiation and bone formation (ALP, type 1 collagen, Runx2, osteocalcin, osteopontin) determined by enzyme immunoassay and PCR[12,15,16,20].

Chondrogenic differentiation of MSCs in vitro

However, other studies have not confirmed the induction of osteogenic differentiation of MSCs on PHBHHx scaffolds[23-25]. It was shown that the spontaneous differentiation of MSCs grown on PHBHHx scaffolds occurs in the chondrogenic direction, which was revealed by a change in the expression of the chondrogenic marker genes of MSCs: *Aggreggan*, *col2*, *sox9*, *col10* and *pthrp*[26]. PHB, PHBV, and PHBHHx scaffolds support chondrogenic differentiation in the chondrogenic medium[27,28]. Chondrogenic differentiation was also demonstrated *in vivo* after implantation of PHB scaffolds seeded with MSCs and was associated with the expression of *sox-9*, *collagen II*, *aggrecan*, *safranin-O*, and glycosaminoglycans[29].

Proliferation of MSCs in vitro

In spite of stimulation of MSC osteogenic differentiation in regular medium, the proliferation of MSCs grown on scaffolds from PHB and PHBV can both have a higher[18,20,21,30] or lower rate[15,17,28,31] in comparison with controls (*e.g.*, tissue culture plastic) in contrast to the suppression of MSC proliferation in osteogenic or chondrogenic media where MSC differentiation was pronounced[17-20]. PHBHHx scaffolds contribute to increasing cell growth[23,24,30,32] but can also cause apoptosis[33] (Table 1).

Other types of MSC differentiation in vitro

Moreover, in neurogenic medium PHB[35,37], PHBV[37], PHBHHx[34,36,37], PHBVHHx[34,36], poly(3-hydroxybutyrate-co-3-hydroxyoctanoate)[35], PHB4HB[37], and their composites support the differentiation of MSCs in the neurogenic direction *in*

Table 1 The effect of natural poly(3-hydroxyalkanoates) on the growth and differentiation of stem cells

The type of PHA	Surface topography/modification, 3D-scaffolds microstructure	The type of stem cells	The time of cell cultivation	The effect of PHAs on the proliferation of stem cells	The effect of PHAs on the morphology of stem cells	The effect of PHAs on the differentiation of stem cells	Ref.
Osteogenic differentiation							
PHB (M_w = 150000) doped with PEG 1000	Porous flat and cubic scaffolds produced by salt leaching from polymer solution using sucrose crystals and ammonium carbonate as porogens	Rat bone marrow MSCs	1, 3, 6, 14 d (proliferation tests); 6, 14 d (differentiation tests)	Suppressed cell proliferation in comparison with TCPS (XTT assay)	Good quality of cell adhesion and spread with developed filopodia (SEM)	Spontaneous osteogenic differentiation in regular medium (increased ALP activity on the 7th and 14th days by up to 10 times and CD45 expression in comparison with TCPS) (ALP activity assay, immunocytochemistry using flow cytometer)	[14,28]
PHBV (with 5 mol % 3-HV)	Porous flat scaffolds produced by salt leaching from polymer solution using sucrose crystals as porogens; oxygen plasma treated and untreated	Rat bone marrow stromal osteoblastic cells (rat bone marrow MSCs)	7, 14, 21, 28 d	Low cell proliferation (60% at day 7 for untreated scaffolds). Treatment with oxygen plasma slightly increased (up to 50%) cell growth (MTS)	-	Spontaneous osteogenic differentiation in regular medium (increased ALP activity on the 28th day by up to 10 times). Treatment with oxygen plasma did not change this effect (ALP activity assay)	[22]
PHBV (with 8 mol % 3-HV)	Porous flat scaffolds produced by salt leaching from polymer solution using sucrose crystals as porogens; oxygen plasma treated and untreated	Rat bone marrow stromal osteoblastic cells (rat bone marrow MSCs)	7, 14, 21, 29, 60 d	Low cell proliferation (2.2 times at day 60 for untreated scaffolds). Treatment with oxygen plasma slightly increased (up to 23%) cell growth (MTS)	Spindle shaped cells on the 29th day with cytoplasmic extensions; large ovoid cells with osteoblast-like morphology on the 60th day. Mineralization from day 21 to day 60. A close connection between the cell boundary and the scaffold (SEM, histology, CM)	Spontaneous osteogenic differentiation in regular medium (increased ALP activity and osteocalcin expression at day 60 by up to 12 times and 4 times, respectively). Treatment with oxygen plasma did not change the induction of ALP activity or increase the induction of osteocalcin expression (ALP activity assay, test for osteocalcin)	[17]

PHBV	Porous scaffolds produced by freeze-drying	Human adipose-derived MSCs	2, 7, 14, 21, 28 d	Increased cell proliferation from days 7 to 28 (MTT)	Good quality of cell adhesion and spread with developed cytoplasmic extensions (SEM)	Signs of spontaneous osteogenic differentiation in regular medium (slightly increased ALP activity at day 28 by up to 10 times). Inhibition of osteogenic differentiation (ALP activity assay) or not (proteins expression assays) in osteogenic medium (osteopontin, collagen I type, osteocalcin indirect immunofluorescence)	[12]
PHB	Porous cubic scaffolds produced by salt leaching from polymer solution using sucrose crystals as porogens	Rat bone marrow and adipose-derived MSCs	3, 7, 14, 21 d (proliferation tests); 7, 14, 21 d (differentiation tests)	Low cell proliferation (up to 2 times at day 21) (MTT assay)	Spherically shaped cells grouped within cell clusters at day 21; calcium deposition (SEM)	Signs of spontaneous osteogenic differentiation in regular medium (a very slight increase in ALP activity and osteocalcin expression at day 21 by up to 4 times); osteocalcin expression; and calcium deposition at day 21 (ALP activity assay, von Kossa staining, PCR for osteocalcin).	[15]
PHBV (with 12 mol % 3-HV) doped with poly(ethylene oxide) (M_w ≈ 1000000) with a mass ratio of 9:1	Scaffolds produced by electrospinning with randomly oriented nanofibers	Rat bone marrow MSCs	1, 4, 7 d (proliferation tests); 7, 14 d (differentiation tests)	Relatively high cell proliferation rate (up to 3-fold at day 7 (CCK-8 test)	Good quality of cell spread in random directions, filopodia extend along the fibers (SEM)	Slight signs of spontaneous osteogenic differentiation in regular medium (a very slight increase in ALP expression at day 14) (ALP staining, PCR), or no effect (staining and expression of osteocalcin, calcium deposition)	[16,21]
PHB, PHBHHx (M_w = 470000)	Porous flat scaffolds produced by salt leaching from polymer solution using salt crystals as porogens	Rabbit bone marrow MSCs	10 d	Higher proliferation in comparison with PLA (MTT assay)	Typical osteoblast phenotype, calcium deposition at day 10 (SEM)	Spontaneous osteogenic differentiation in regular medium at day 10 (ALP activity assay)	[18]
PHBV (with 5 mol % 3-HV)	Films casted from polymer solution	Murine calvarial preosteoblast cells MC3T3-E1 S14 line	21 d	-	A dense monolayer of cuboidal-shape cells with obvious areas of mineralization (CM)	Higher stimulation of osteogenic differentiation in comparison with cells grown on TCPS in osteogenic medium (Calcium C test, von Kossa staining)	[19]

PHBV (with 12 mol % 3-HV; M_w = 530000)	Scaffolds produced by electrospinning with randomly oriented fibers	Human bone marrow MSCs	1, 7, 11 d (proliferation tests); 7 d (differentiation tests)	No difference between cell proliferation on scaffolds from PHBV and poly-ε-caprolactone (trypan blue assay)	Good quality of cell adhesion and spread with developed filopodia. Cells have a nearly spherical shape (CM)	Higher modulation of osteogenic differentiation in comparison with MSCs grown on poly-ε-caprolactone scaffolds in osteogenic medium (calcein and Alizarin red staining assay)	[13]
PHBV (with 5 mol % 3-HV, M_w = 680000)	Scaffolds produced by electrospinning with randomly oriented nanofibers	Human induced pluripotent stem cells	1, 3, 5, 7, 10 d (proliferation tests); 7, 14 d (differentiation tests)	The higher proliferation rate in comparison with TCPS (MTT assay)	Original-like MSCs formed colonies at day 5 (LM).	Higher stimulation of osteogenic differentiation in comparison with cells grown on TCPS in osteogenic medium (ALP activity assay, RT-PCR for measure of ranx-2, col-1, ALP, osteonectin, osteocalcin expression levels; Western blot assay for osteocalcin and osteopontin expression levels)	[20]
PHBHHx (with 12 mol % 3-HHx), and PHBVHHx	Films casted from polymer solution	Human bone marrow MSCs	4, 72 h (proliferation tests); 14, 21 d (differentiation tests)	Higher cell proliferation rate (for PHBHHx and PHBVHHx) in comparison with PLA and with TCPS (for PHBVHHx)	-	No effect on cell differentiation in regular medium (FM: ALP and van Kossa staining)	[23]
PHBHHx (with 8.3 mol % 3-HHx, M_w = 1210000)	Smooth compression-molded films, porous scaffolds casted from solution films, electrospun scaffolds	Human bone marrow MSCs	5, 6, 7, 14 d (proliferation tests); 14 d, 5 wk (differentiation tests)	The same (for compression-molded films) and higher (up to 2-fold for solution-casted and electrospun films) cell proliferation in comparison with TCPS	Spindle-like, similar to original MSCs, good cell adhesion and spreading in regular medium (FM, SEM).	No differentiation in regular medium. Inhibition of differentiation in osteogenic medium (osteocalcin assay, Alizarin red S staining, RT-PCR for measure of collagen I and osteonectin expression level)	[24]
PHBHHx (M_w = 300000)	Scaffolds produced by electrospinning with randomly oriented fibers	Rat bone marrow MSCs	3 d	-	The well-developed stress fibers spanned the entire cell body and supermature focal adhesions (CM, immunofluorescence)	No osteogenic differentiation: no significant expression of osteocalcin, osteopontin, osteonectin, *runx2*, in regular and osteogenic media at day 3 (RT-PCR)	[25]
Chondrogenic differentiation							

PHB and PHBHHx in a ratio of 1:2 (by weight)	Porous flat scaffolds with pores of 200–300 μm in diameter produced by salt leaching from polymer solution using salt crystals as porogens and lyophilization	Human adipose-derived stromal cells	7 d (proliferation tests); 14 d, 5 wk (differentiation tests)	100% cells viability at day 7 (FM)	After 1 d, the differentiated cells attached to scaffolds. At 7 d and 14 d, the differentiated cells produced extracellular matrices to fill the voids of the scaffolds (SEM)	Chondrogenic differentiation in chondrogenic medium: increased glycosaminoglycan and collagen content. No chondrogenic differentiation in regular medium: a very slight increase in collagen content (biochemical glycosaminoglycan and collagen content assays)	[27]
PHBHHx (M_w = 600000)	Films casted from polymer solution	Murine bone marrow MSCs; chondrocytes isolated from cartilage from knees of mice	1 d (24 h)	No change in cell proliferation in comparison with TCPS (RT-PCR analysis of proliferating cell nuclear antigen)	-	Spontaneous chondrogenic differentiation in regular medium at day 1 (as in chondrocytes with the exception of *col1*): upregulation of *aggrecan*, *col2*, *sox9*, *col10*, *pthrp*, and *col1* genes, downregulation of osteocalcin, Cbfa1/Runx2, MMP13 genes, microRNAs miR-29a and miR-29b (alcian blue staining for glycosaminoglycans; immunostaining for type II collagen; RT-PCR analysis of chondrogenic markers)	[26]
PHBV	Porous cylindrical (5 mm diameter, 2 mm thick) scaffolds with a pore size of 30-300 μm produced by salt leaching	Swine bone marrow MSCs, cartilage progenitor cells, and chondrocytes	After 1 wk of *in vitro* culture subcutaneous implantation *in vivo* into nude mice for 6 wk.	Higher wet weight and volume of the cell-scaffold constructs seeded with cartilage progenitor cells and chondrocytes in comparison with MSCs 6 wk after implantation.	Good compatibility between the cells and the scaffold and production of considerable amounts of extracellular matrix after 1 wk of *in vitro* culture (SEM)	Higher chondrogenic differentiation *in vivo* of cartilage progenitor cells and chondrocytes: increased expression of *sox-9*, *collagen II*, *aggrecan*, *safranin-O*, glycosaminoglycans in comparison with MSCs (alcian blue staining, immunostaining, RT-PCR)	[29]

PHBHHx	Films casted from polymer solution	Human umbilical cord MSCs	3, 7, 14 d (proliferation tests); 14, 28 d (differentiation tests)	Higher MSCs proliferation in chondrogenic medium in comparison with TCPS (MTT assay)	Good spreading of cells and their proliferation (SEM)	Chondrogenic differentiation in chondrogenic medium: increased glycosaminoglycan, proteoglycan, and collagen content, upregulation of *collagen II* and *aggrecan*, genes (amino-sugars and hydroxyproline assays, toluidine blue staining, RT-PCR)	[28]
Epidermal differentiation							
PHBV, (M_w = 450 kDa)	Scaffolds produced by electrospinning with randomly oriented nanofibers	Bone marrow MSCs	1, 3, 7, 14 d (proliferation tests); 3, 7, 14 d (differentiation tests)	100% viability of cells. Higher cell proliferation in epidermal-induction medium in comparison with regular medium. Lower cell proliferation on PHBV scaffolds in regular and epidermal-induced medium in comparison with TCPS (CM, MTS assay)	Good adhesion and spreading of cells tended to start forming a monolayer at day 7. The spindle-like, fully extended morphology of MSCs at day 3. The keratinocytic morphology of MSCs characterized by polygonal cells at days 7 and 14 (SEM, CM)	The epidermogenic differentiation in epidermal-induction medium: increased expression of keratin, filaggrin, and involucrin. No epidermogenic differentiation in regular medium (CM, RT-PCR)	[38]
Adipogenic differentiation							
PHBHHx (M_w = 300000)	Scaffolds produced by electrospinning with randomly oriented fibers	Rat bone marrow MSCs	3 d	-	Well-developed stress fibers spanned the entire cell body and supermature focal adhesions (CM, immunofluorescence)	No adipogenic differentiation: no significant expression of *PPARg*, *Lpl*, *ADFP*, *CD36* in regular and adipogenic media at day 3 (RT-PCR)	[25]
Endotheliogenic differentiation							
PHB/PHBV composite (30:70)	Scaffolds produced by electrospinning with randomly oriented nanofibers	Human adipose tissue-derived MSCs	7, 14, 21 d (proliferation tests); 7, 14, 21 d (differentiation tests)	Lower cell proliferation on scaffolds in endothelial-induction medium in comparison with TCPS. Higher cell proliferation on scaffolds in regular medium in comparison with TCPS (CM, MTT assay)	Good adhesion and spread, typical spindle-shape morphology, and cell-to-cell interactions. Good distribution of cells in regular medium. MSCs in endothelial-induction medium formed circle-like structures characteristic of endothelial cell organization, mimicking the tubular organization of blood vessels at day 21 (SEM, CM, calcein-AM staining)	Endotheliogenic differentiation in endothelial-induction medium: increased expression of VE-Cadherin, vWF factor, and VEGFR2 (immunostaining, flow cytometry, RT-PCR)	[39]
Neurogenic differentiation							

PHBHHx and PHBVHHx	Films casted from polymer solution, porous scaffolds with a pore size of 110-170 μm produced by in solution phase separation	Human bone marrow MSCs	2, 3 d (proliferation tests); 7, 14, 21 d (differentiation tests)	Slightly higher cell proliferation on PHBHHx and PHBVHHx films (33% and 31%, respectively) in comparison with PLA films in regular medium at day 3 (CCK-8 assay)	Good adhesion, spread, and proliferation in PHBHHx and PHBVHHx films and PHBVHHx scaffolds in regular medium (SEM, CM)	Neurogenic differentiation of neural stem cells in neurogenic medium: increased expression of nestin, β-tubulin III and anti-glial fibrillary acidic protein (slightly higher expression levels of these markers in cells grown on PHBHHx and PHBVHHx films in comparison with PLA)	[34]
PHBHHx and PHBVHHx	Films casted from polymer solution	Rat neural embryonic stem cells	1, 3, 5 d (proliferation tests); 3, 7 d (differentiation tests)	Same cell proliferation and viability on PHBHHx and PHBVHHx films in comparison with PLA films in neurogenic medium (CCK-8 assay)	Bipolar or even monopolar morphology of the cells with relatively short neuritis at day 3 in neurogenic medium (CM)	Neurogenic differentiation of neural stem cells in neurogenic medium: increased expression of β-tubulin III and anti-glial fibrillary acidic protein (the same level of this marker expression in cells grown on PLA, PHBHHx and PHBVHHx films)	[36]
PHB, PHBV, P3HB4HB, and PHBHHx	Films casted from polymer solution, porous scaffolds produced by in solution phase separation and freeze-drying	Rat neural embryonic stem cells	7, 10 d (proliferation tests); 7, 14, 21 d (differentiation tests)	Slightly higher cell proliferation on PHB, PHBHHx and PHB4HB films in comparison with PLA films in neurogenic medium (CCK-8 assay)	Cells with extended processes and plausible neurite connections at days 3 and 7 in neurogenic medium (CM, SEM)	Neurogenic differentiation of neural stem cells in neurogenic medium: increased expression of β-tubulin III (higher expression level of this marker in cells grown on PHB4HB and PHBHHx in comparison with PHB)	[37]

SEM: Scanning electron microscopy; CM: Confocal microscopy; FM: Fluorescence microscopy; LM: Light microscopy; RT-PCR: Reverse transcription polymerase chain reaction; TCPS: Tissue culture plastic; ALP: Alkaline phosphatase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; PHB: Poly(3-hydroxybutyrate); PHBV: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PHBHHx: Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); MSCs: Mesenchymal stem cells; PHAs: Poly(3-hydroxyalkanoates); PLA: polylactic acid.

vitro, which results in a change in cell morphology and expression of genes of specific proteins: Nestin, glial fibrillary acidic protein, and βIII-tubulin[34-37]. The differentiation of MSCs in the epidermal (to keratinocytes) and endothelial (to vascular endothelial cells) directions was also demonstrated in appropriate special stimulating media using nanofibrous scaffolds produced by electrospinning as a substrate for cell growth[38,39].

The effect of PHAs on tissue regeneration

The osteoinductive properties of PHB and its copolymers were displayed not only *in vitro* but also *in vivo* when scaffolds derived from these polymers were used as bone-substituting biomaterials and were implanted into bone defects. PHB, the main polymer of a homologous series of the family of PHAs, is the best-known microbiological polyester, which is a promising alternative to biodegradable synthetic thermoplastics and is actively used in regenerative medicine and tissue engineering. The biodegradation rate of PHB and its copolymers is much lower than the biodegradation rate of synthetic PLA, PGA, and their copolymers with the very

intensive process of bone tissue reconstruction, which makes PHB use for the regeneration of bone tissue more relevant. Polymer biodegradation mainly occurs due to the phagocytic activity of specialized cells (macrophages, foreign body giant cells, and osteoclasts), *i.e*. specialized cellular biodegradation of these biopolymers takes place. The greater biocompatibility of PHB and its copolymers compared with synthetic PHAs due to the absence of the effect of acidification of surrounding tissues by the polymer biodegradation products is also of great importance. When the tissue reaction to PHB was compared with tissue reactions to synthetic PLA, PGA polyesters, and their copolymers, several studies found mild or moderate tissue reactions to PHB, while severe chronic inflammatory reactions were not infrequently observed for PLA, PGA, or PLGA[8,11].

Porous scaffolds and patches from PHB were shown to promote bone tissue regeneration, which was demonstrated in experimental models of critical (parietal bone of the rat skull) and noncritical (rat femur) bone defects. At all stages of the process of bone defect regeneration, a minimally pronounced tissue reaction to implantation was observed. This reaction was associated with the gradual bioresorption of the polymeric material by osteoclasts, active vascularization of the scaffolds, and sprouting of the newly formed bone tissue into the pores of the PHB scaffolds[8,11]. Regeneration of bone tissue in the PHB scaffolds was also indicated by the elevated expression of osteogenic markers, *e.g*., collagen type I[15,40]. or their expression was in opposite reduced[41]. We observed the appearance of the newly formed bone tissue evenly throughout the volume of the porous biopolymer scaffolds in the form of islands, not from its edges, while a fibrous capsule was not formed around the material, indicating complete integration of the biopolymer material with the bone tissue[42]. Moreover, it was shown that other bone tissue devices based on PHB (nonwoven patches, porous scaffolds) could contribute to the regeneration of other tissues of various organs, including nerves, intestine, heart, and vessels. A high level of vascularization (angiogenesis) and the increased expression of vascular endothelial growth factor were shown in the tissue defect area during regeneration using PHB devices[8,11,15,41]. It should be noted, however, that PHB and its copolymers can activate immune cells during implantation causing the secretion of proinflammatory cytokines by these cells[43,44], which is a typical tissue reaction to implantation of virtually any material.

The problem of the interaction of PHAs and MSCs is closely related to the use of these polymers for the manufacture of medical devices for regenerative surgery and tissue engineering of the intestine using devices from synthetic polyhydroxyalkanoates. The successful transplantation of tubular prostheses on the basis of PGA nonwoven material was carried out to replace a dog esophagus. For this purpose, the prostheses were seeded with fibroblasts and keratinocytes and placed in the abdominal cavity to develop the tissue engineering construction *in situ*. Development of the mucosa and muscular membrane was observed after the esophageal substitution, while the prosthesis that was not seeded with cells was rejected[45].

Successful use of the tissue engineering construction based on a surgical mesh fabricated of PGA with a PLA cover as a patch for the stomach wall was demonstrated in a model of stomach wall defects in rats. The surgical mesh was seeded with embryonic epithelial cells, and patch fusion with the stomach wall tissues and the development of mucous and smooth muscle membranes were demonstrated[46]. Japanese researchers developed a tissue engineering construction based on a porous tubular prosthesis from PGA seeded with embryonic epithelial intestine cells. In rats, the anastomosis of a tubular prosthesis with rat jejunum was surgically generated to analyze the regenerative potential of the construction, and the development of mucosa with villous epithelium on a polymer scaffold and its integration with the intestine wall tissues were observed[47]. With improvements in the endoscopic suturing technique, new opportunities for the application of tissue engineering techniques arise for the regeneration of gastrointestinal tract tissues; good results have already been demonstrated, proving the techniques to be safe and reliable. Takeshita *et al*[48] successfully used a poly(6-hydroxycaprolactone) scaffold produced by 3D printing and seeded with fibroblasts to close intestinal wall defects by an endoscopic suturing technique.

Intestinal progenitor stem cells are intensively used for tissue engineering of the stomach, small intestine, and bowel in combination with polymer scaffolds[49,50]. However, investigation of intestinal stem cells on scaffolds from PHAs or even synthetic polyhydroxyalkanoates or the development of tissue-engineered devices based on PHA scaffolds seeded with intestinal stem cells are very rare. A good example of such rare studies of particular interest is the work of Costello CM[51] and Shaffiey SA[52]. Their porous scaffolds based on PLGA of a complex microarchitecture (modeling the small intestine mucosa villi) were developed for tissue engineering of the intestinal mucosa. The active growth of the intestinal epithelial cells was

demonstrated on the obtained scaffolds using both the commercial *Caco-2* line and cells isolated from the human intestinal mucosa. The development of villus-like, polymer-cellular hybrid structures, the differentiation of cultivated cells in functional cells of the intestinal mucosa (enterocytes, goblet cells, and Paneth cells), and mucus production was demonstrated[51]. Then, the researchers developed a tissue-engineered construction of such a complicated structure seeded with coculture of intestinal stem cells, myofibroblasts, macrophages, and probiotic bacteria and demonstrated enhanced mucosal regeneration in the dog rectum upon using this construction[52].

ROLE OF PROPERTIES OF PHAS AS NATURAL BIOPOLYMERS IN THEIR EFFECT ON MSC GROWTH AND DIFFERENTIATION

The role of the microstructure in the effect of PHAs on MSC growth and differentiation

The microstructure and topography of the surface on which cells grow can play particularly important roles in the growth and differentiation of MSCs. Moreover, these features can even reverse the effects of bioactive molecules on cell growth or differentiation[24,25,53]. The effect of the biological activity of polymers on the growth and differentiation of MSCs both *in vitro* and *in vivo* depends on the microstructure of 3D devices made from them: surface topography[24], 3D-microstructure[25], porosity, pore size, and shape[9,54]. Different cells prefer different surfaces. For example, MSCs and osteoblasts prefer rougher surfaces with the appropriate size of pores[55,56], while fibroblasts prefer smoother surfaces, and epithelial cells attach only to the smoothest surface[57]. This appropriate roughness affects cell attachment because it provides the right space for the growth of MSCs or supplies solid anchors for their filopodia. A scaffold with the appropriate size of pores provides better surface properties for anchoring type II collagen filaments and for their penetration into internal layers of the scaffolds seeded with chondrocytes, which is supported by the interaction of extracellular matrix proteins with the material surface[24,25].

However, the study of the proliferation and differentiation of MSCs grown on electrospun scaffolds with randomly oriented and aligned fibers yields contradictory data: the aligned fibers stimulate the osteogenic differentiation of MSCs[25], or there was no significant difference in cell differentiation compared with randomly oriented fibers[16]. Moreover, scaffolds with porous surfaces can inhibit the osteogenic differentiation of MSCs cultivated in the osteogenic medium[24]. The appropriate surface properties may also promote cell attachment and proliferation by providing more spaces for better gas/nutrient exchange or more serum protein adsorption[9,54].

The scaffolds from PHAs, which are manufactured by a series of techniques (salt leaching, electrospinning, phase separation, blending, *etc*) have a fibrous porous microstructure, which is a biomimetic structure similar to the extracellular matrix[58]. Surprisingly, PHAs are easily processed by various methods to produce such biomimetic structures (Table 1). It may also be related to the natural properties of PHAs, such as the complex structure of bacterial carbonosomes, where the synthesis of PHB is carried out[59].

The role of physicochemical properties in the effect of PHAs on MSC growth and differentiation

It is generally accepted that the effect of PHAs on the growth and differentiation of MSCs is connected with the physicochemical properties of the polymeric material and the 3D and surface microstructure of scaffolds made from this polymer. Indeed, the chemical composition, hydrophilicity, and charge of the polymer surface have significant influences on cell attachment, viability, and proliferation[60]. For example, physical or chemical modifications of the surface of PHA devices can improve the attachment and growth of cells on the surface. Lipase treatment increases the viable cell number on PHB films by 100-200 times compared with untreated PHB film. Treatment of PHB films with NaOH also led to a 25-fold increase in the viable cell number compared with untreated PHB film[61]. It was shown that treatment of PHB film surfaces with low-pressure ammonia or oxygen plasma improved the growth of human fibroblasts, epithelial cells of respiratory mucosa, and rat bone marrow stromal osteoblastic cells (rat bone marrow MSCs) due to increased hydrophilicity (but with no change in microstructure) of the polymer surface[17,22,62]. It was suggested that the improved hydrophilicity of the films after PHB treatment with lipases, alkali, and plasma could allow cells in suspension to more easily attach to the polymer films compared with untreated films. The influence of the hydrophilicity of the biomaterial

surface on cell adhesion was demonstrated previously[63]. Some researchers used PHAs only as a framework to cover biomaterials and investigated the effect on the growth and differentiation of MSCs. Thus, collagen type I, containing or not containing chondroitin sulfate, was covered on the textile scaffold from PHB threads, and the osteogenic differentiation of human MSCs isolated from bone marrow was studied[64].

Surprisingly, some PHAs (including PHB and PHBV) have piezoelectric properties similar to those of natural bone. Mechanical stress in bone tissue produces electrical signals that stimulate bone growth and remodeling[13]. Electrical stimulation resulted in significant increases in the osteogenic differentiation of MSCs *in vitro* and improved the regeneration of critical bone defects *in vivo*[65]. Thus, the osteogenic activity of PHB and its copolymers could be due to the piezoelectric properties of these biopolymers.

The physicochemical properties of PHAs are related entirely to the function of natural PHAs as storage polymers in a bacterial cell. The relative hydrophobicity of PHB is due to the elimination of the effect of high osmotic pressure on the storage polymer[6,7,10]. The semicrystalline structure and thermoplasticity of PHAs are connected with the necessity to maintain the particular state of the polymer in the cell, which should be simultaneously stable in water medium and available for controlled digestion by PHA depolymerases. Chemical inertness is the main reason for PHA biocompatibility and is associated with the nontoxicity of storage polymers for bacterial cell[6,10,66].

The role of biodegradation as a natural property in the effect of PHAs on MSC growth and differentiation

The ability of PHAs to biodegrade may be the stand-alone reason for the biological activity of these polymers, including the relation with MSCs. As biodegradable polymers, natural PHAs cause targeted activation of macrophages and osteoclasts – cells that directly cause biodegradation of the polymers. It was shown that macrophages and osteoclasts adhere strongly to and proliferate on polymer films. On the polymer surface, macrophages are activated by a polymeric material, which contributes to cell phagocytic activity[67,68]. The adhesion of macrophages to the surface of a polymeric material plays a key role: it was shown that biodegradation of polymeric membranes occurs only when macrophages adhere to their surface, and if macrophages cannot adhere to the membrane, the polymer does not degrade[68].

Macrophages can uptake microparticles from low-molecular PHB with a size of 1-10 μm by phagocytosis[67]. At high concentrations of PHB particles (> 10 μg/mL), phagocytosis is accompanied by toxic effects and changes in the functional state of macrophages[67], while nanoparticles (15-250 nm) of PHB and its copolymers do not show significant cytotoxic effects on macrophages even at a high concentration of 1 mg/mL unlike nanoparticles from PLA[69]. Phagocytosis of microparticles from PHB is accompanied by an increase in the production of nitric oxide and tumor necrosis factor alpha in activated macrophages. Macrophages died after the uptake of a large number of microparticles. It was also demonstrated that phagocytosis of particles from PHB decreases with time due to the active process of PHB biodegradation[67].

In addition to macrophages, fibroblasts and osteoblasts are also capable of uptaking PHB microparticles *in vitro*. Even at high concentrations of PHB particles (> 10 μg/mL), phagocytosis is not accompanied by toxic effects on fibroblasts[67]. The endocytosis of microparticles by osteoblasts is accompanied by the differentiation of cells in the osteogenic direction as indicated by the increase in ALP activity[70]. The PHBHHx nanoparticles were used to study the mechanism of stimulation of cell proliferation. In concentrations from 0.02 to 0.1 g/L, they stimulated an increase in the calcium ion current in the cell cytoplasm, which is one of the main signaling pathways of activation of their division[71-73].

Oligomers of PHAs may also have their own biological activity. Therefore, it was shown that oligomers of PHB and its copolymers with 4-hydroxybutyrate and 3-hydroxyhexanoate (with a chain length of 20-25 monomers) are not toxic to cells (up to a concentration of 20 μg/mL) and have biological activity. They stimulate proliferation, inhibit apoptosis, and suppress the release of calcium into the cytoplasm and the formation of intercellular contacts between the B cells of the pancreas of mice[74].

The process of biodegradation of material by osteoclasts or macrophages can stimulate MSC differentiation in the osteogenic direction. Active osteoclasts grown on biodegradable ceramic scaffolds were shown to influence the development of MSCs into osteoblasts when cocultured *in vitro* by STAT3 activation[75,76]. Therefore, the ability of PHA to biodegrade itself may be an indirect cause of the induction of MSC differentiation. The ability of hydrolytic and enzymatic degradation (with slow rate) is strongly connected with the need for controlled consumption of this polymer as an energy supply by enzymatic cleavage in special granules of a bacterial cell[6,10,59].

Moreover, the products of PHA biodegradation are common substances in human organisms because of the presence of PHB and its copolymers in bacteria of the human microbiota. A series of symbiotic and infectious bacteria of mammals (including human) of the genuses *Agrobacterium, Clostridium, Ralstonia, Bacillus, Burkholderia, Vibrio, Legionella, Pseudomonas, Mycobacterium, Acinetobacter, Sphingomonas, Fusobacterium, Neisseria, Streptomyces*, and *Bordetella, Rickettsia* can synthesize this biopolymer or have the enzymes (or their genes) of PHB biosynthesis, PHA-polymerase.

Some of these bacteria, such as *Pseudomonas sp*. are capable of synthesizing not only PHB but also various PHB copolymers[66]. The role of this biopolymer in the bacteria of animal microbiota remains a very poorly understood problem. However, a number of papers have suggested the participation of PHB in the relationship between bacteria and the host organism. Kim *et al*[77] suggested that the PHB synthesis facility allows symbiotic bacteria of the *Burkholderia* genus to survive in the intestine of the *Riptortus pedestris* bean bug in the stress conditions induced by the host organism immune system to regulate the number of these bacteria. Apparently, PHB synthesis by bacteria from *Rhodobacterales* modulated the gastrointestinal tract microbiota in sea cucumbers *Apostichopus japonicus* contributing to an increase in the animal size multiple times[78].

Moreover, the efficiency of PHB in the fight against infectious diseases in animals was demonstrated: the use of PHB powder as an additive to feed protected *Artemia nauplii* crustaceans from infectious disease caused by pathogenic *Vibrio campbellii* bacteria. The efficiency of PHB was 100 times larger than that of the 3HB monomeric precursor[79]. In addition, PHB has the ability to suppress pathogenic bacteria, not only *Vibrio sp*. but also *E. coli* and *Salmonella sp*.[80]. PHB oligomers (with a length of approximately 80 3HB monomers) possess effective concentration-dependent antibacterial and antifungal properties against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans* through disruption of the bacterial wall/membrane and changes in the transmembrane potential[81].

At the genus level, dietary PHB increased the abundance of beneficial bacteria in the intestine of Pacific white shrimp *Litopenaeus vannamei*, such as *Bacillus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, and *Bdellovibrio*. This beneficial effect was associated with activation of the rapamycin (mTOR) signaling pathway, which plays a crucial role in intestinal inflammation and epithelial morphogenesis[82]. However, the mTOR signaling pathway is involved in MSC proliferation and differentiation. Suppression of the mTOR signaling pathway enhances the osteogenic capacity of stem cells, while mTOR activation causes MSC hyperproliferation[83,84].

Moreover, the modified products of PHB biodegradation methyl-esterified dimers and trimers have pronounced antioxidant activity: 3-fold greater hydroxyl radical–scavenging activity than glutathione and 11-fold higher activity than vitamin C or 3HB monomer[85]. Unfortunately, the role of possible antioxidant properties in biocompatibility or biological activity of PHAs is unexplored, while it can be also be connected with their effect on MSCs. Oxidative stress is one of the regulatory mechanisms for MSC osteogenic and adipogenic differentiation. For example, antioxidant vitamin D3 promotes the osteogenic differentiation of MSCs under normal conditions and partly protects cells from oxidative stress damage by activating the endogenous antioxidant system[86,87]. Maybe this is one of the main reasons for PHA biocompatibility, especially with stem cells. But if PHAs have their antioxidant properties as their natural functions, *e.g*., in bacterial cells[85], it can explain the mechanism of interaction of PHAs with stem cells.

However, the possible effect of intestinal microbiota bacteria on PHA polymeric devices (and the effect of the polymeric material of these devices on symbiotic bacteria) is not always taken into account when polyhydroxyalkanoates are used to regenerate intestinal tissues. In particular, the roles of natural PHAs and the degradation products in the interaction with intestinal progenitor stem cells and microbiota bacteria remain entirely unexplored. However, the fundamental roles and functions of PHB in relation to human intestinal microbiota bacteria should be taken into account in the development of medical devices based not only on PHB and other natural PHAs but also on their biomimetic analogs, such as synthetic polyhydroxyalkanoates, especially devices based on these polymers for intestine surgery.

The role of 3-hydroxybutyrate in the effect of PHAs on MSC growth and differentiation

The effect of PHAs on the differentiation of MSCs can also be associated with the bioactivity of 3HB that is produced during PHB and its copolymer biodegradation as the primary monomeric precursor of these polymers. Thus, 3HB at a concentration of 0.01 to 0.1 g/L (0.1-1 mmol/L) can cause activation of the proliferation of human

keratinocytes of the *HaCaT* line and mouse *L929* fibroblasts by increasing the concentration of calcium ions in the cell cytoplasm and by inhibiting the apoptosis and necrosis of fibroblasts[71-73]. Such bioactivity of 3HB is not surprising because 3HB is a natural metabolite in the mammal body. It is the so-called ketone body with a pronounced versatile biological activity. For example, 3HB performs regulatory functions under various special conditions such as diabetes, starvation, and a ketogenic diet[88,89]. 3HB at a concentration of 0.005-0.1 g/L (0.05-1 mmol/L) induced osteogenic differentiation of mouse MC3T3-E1 preosteoblast cells (unfortunately, not MSCs), which was determined by an increase in ALP activity, calcium deposition (Alizarin red S dye assay), and osteocalcin expression. It should be noted that PHAs enhanced osteogenic differentiation of both MSCs and exactly this cell line of preosteoblast cells.

Moreover, it was demonstrated that 3HB has *in vivo* osteoinductive activity that was shown in an osteoporosis model in female rats with removed ovaries. However, at lower concentrations, 3HB did not have a similar effect. For slower PHA biodegradation, 3HB formed at concentrations much lower than 0.05 mmol/L[90]. It was also shown that 3HB stimulates the formation of gap junctions between neurons for the transmission of electrical signals, which is associated with improved memory and learning[91]. The ability of PHAs to cause neurogenic differentiation of MSCs could also be related to the previously shown neuroprotective effect of 3HB. 3HB manifests itself at very high doses because the effect of 3HB on the nervous system is due to the nutrient (energy) function of fatty acids for neurons[92].

Furthermore, if PHAs cause differentiation of MSCs only by 3HB biological activity, it is difficult to explain the data of a series of investigations in which the growth and differentiation of MSCs were compared among scaffolds fabricated of different PHAs (PHB and its copolymers usually). The effect of different PHAs on the growth and differentiation of MSCs differs significantly and even dramatically, while the content of 3HB monomers in PHB copolymers is rarely lower than 80 mol %[18,23,33,93]. It was shown that 3HB functions through multiple mechanisms: inhibition of class I histone deacetylases; binding and activation of cell surface G-protein-coupled receptors, hydroxycarboxylic acid receptors, and free fatty acid receptor 3; histone modification by covalent binding with proteins (β-hydroxybutyrylation); and membrane channel and transporter regulation[89]. The product of biodegradation of synthetic PLA and PLGA - L-lactate (2-hydroxypropanoate) was shown to be a natural ligand for Gi-coupled G-protein receptor 81 inhibiting cAMP-mediated intracellular signaling events such as lipolysis[94].

It was also revealed that the biodegradation products of some natural PHAs (*e.g.*, 3-hydroxyoctanoate) have antimicrobial activity against a number of infectious gram-negative and gram-positive bacteria. They inhibit the production of metabolites associated with their pathogenic activity. However, their cytotoxic activity against human fibroblasts is manifested at much higher concentrations[86].

Short-chain fatty acids (including 3HB and L-lactate) are the critical metabolites of mammalian microbiota by which symbiotic bacteria execute the diet-based microbial influence on the host. They affect various physiological processes in the gastrointestinal, nervous, and immune systems and may contribute to health and disease (colitis, diabetes, cancer, asthma)[95]. Thus, the observed effect of 3HB on MSC growth and differentiation could be associated with one of the mechanisms of microbiota influence on mammalian host organisms.

The possible role of natural signaling functions of PHB in the effect of PHAs on MSC growth and differentiation

Notwithstanding the above, data from a series of studies provide evidence of the biological activity of PHAs toward cells (including MSCs) through receptor or cellular signaling molecule-associated mechanisms[25,26,33,96]. As we can see in Table 1 PHAs induce osteogenic and chondrogenic differentiation and support MSC differentiation in these and other directions: epitheliogenic, adipogenic, endotheliogenic, and neurogenic. It also can change the MSC proliferation rate, which is associated with change in cell morphology, physiological state, extracellular matrix synthesis, and expression of a series of differentiation markers: ALP, collagen I and II types, osteopontin, osteocalcin, glycosaminoglycan, aggrecan, sox-9, *etc*. The possible mechanism of the implementation of the effects of PHAs on cell proliferation, differentiation, and apoptosis can be accomplished through integrins – intercellular macromolecules for contact and recognition. It was shown that MSC differentiation and apoptosis of osteoblasts is carried out by a cascade mechanism that includes regulator proteins such as peroxisome proliferator-activated receptor gamma, which is triggered by the interaction of PHAs with integrins on the cell surface[25,33]. It was also shown that the osteogenic and chondrogenic differentiation of MSCs induced by the polymer surface of PHAs could be regulated by microRNAs[26,96]. The microRNAs

miR-29a and miR-29b were confirmed to be significantly downregulated in MSCs grown on PHBHHx films through cell-material interactions. They can directly target the 3′ UTR of *col2a1* and are suppressed by the chondrogenic protein *Sox9* to induce chondrogenesis in MSCs[26].

Some researchers attribute the biological activity of PHB and its copolymers obtained by bacterial biosynthesis to the fact that the polymer biomaterials may be poorly purified from bacterial lipopolysaccharide or DNA. However, a previous study made clear that even a very well-purified polymer is able to activate the cellular response[68].

The problem of the biological activity of PHAs, *e.g*., their ability to effect stem cell growth and differentiation, seems to be closely related to the problem of endogenous PHB in mammalian tissues. The finding of bacterial PHB in various tissues of mammals was one of the most exciting stages in the history of PHA research. It should be immediately noted that it is not a high-molecular reserve polymer. The synthesis is typical for a number of bacteria, but the so-called short-chain complex-forming PHB (cPHB) and low-molecular oligo-PHB (oPHB) were found[59]. While specific PHB biosynthesis enzymes are available only in prokaryotes, this biopolymer was found in organisms of almost all types. Short- and medium-chain PHBs were found in many different mammalian organs and tissues (including in humans), *i.e*. in the blood plasma, heart, kidneys, liver, vessels (aorta), nerves, lipoprotein particles, platelets, *etc*. It was demonstrated that oPHB is located in eukaryotic cell membranes in the form of a PHB–polyphosphate–calcium complex. The oPHB has a molecular weight of 12200, which corresponds to a length of approximately 140 elements of 3HB. The oPHB concentration in human blood plasma can change in a rather wide range, from 0.6 to 18.2 mg/L at an average value of 3.5 mg/L[97].

Thus, what function does PHB have in the mammalian organism? In addition to the role of PHB as a reserve substance and energy depot in bacteria, the researchers who discovered endogenous PHB in mammalian tissues suggested some of its functions. The functions of endogenous PHB for both prokaryotic and eukaryotic organisms are apparently coupled with the regulation of different proteins due to the production by short-chain cPHB and oPHB of both noncovalent and covalent bonds with other biopolymers (proteins, nonorganic polyphosphates, and DNA). In the form of PHB-Ca-polyphosphate complexes, cPHB can function as nonprotein channels capable of conducting inorganic ions through the plasma membrane; these structures also form noncovalent complexes with channels proteins and become part of them)[97]. It was demonstrated that cPHB binds to one of the proteins from the group of melastatin receptors (*TRPM8*) of mammals (and humans), which leads to a change in its functioning[97,98]. *TRPM8* is a membrane calcium channel that functions as a temperature sensor of neurons of the mammalian peripheral nervous system. The binding of the protein *TRPM8* receptor channel with cPHB is required for normal functionality of this receptor, which is associated with a change in the conformation state of PHB when passing through this polymer's glass transition temperature, which is approximately 10 °C[59,97,98].

Another case of the modification of proteins is the *OmpA* protein of *E. coli*. The covalent attachment of cPHB to the specific serine residues of *OmpA* maintains the correct integration and orientation of this protein in the outer membrane of the bacterium. It was proposed that such modification of proteins by cPHB involves the folding and sorting of certain proteins[99]. Similar processes have been described for mammals. Thus, it was shown that PHB oligomers are covalently bound to the calcium ATPase of the plasma membrane of human erythrocytes, simultaneously forming a complex with inorganic phosphates[100].

Other researchers have indirectly confirmed that such conjugation of cPHB with proteins may have a specific physiological function. It was shown that conjugation of the antitumor peptides DP18L with 3-hydroxydecanoate enhances their antitumor activity[101,102]. The formation of covalent bonds of PHB with proteins during biochemical processes can be indirectly confirmed by the ability of many bacterial strains, producers of high-molecular-weight PHB, to utilize other polymers added to the culture medium, which often differ significantly in their physicochemical properties (for example, hydrophilic PEG) by covalent crosslinking with the end of the synthesizing PHB chain and the formation of diblock copolymers (for example, PHB-PEG)[103].

There is a striking example of other biopolymers with such functionality that, in addition, are quite similar to PHB in chemical structure: polyprenols (in bacteria, plants, and fungi) and dolichols (in bacteria and animals). Polyprenylation (covalent binding to polyprenols) plays an essential role in the posttranslational modification of a number of proteins to anchor them in the membrane. All organisms (both eukaryotes and prokaryotes) have biochemical mechanisms for the synthesis of these biopolymers and their conjugation with proteins[88]. Appropriately, there is evidence

suggesting that the functionality of cPHB and oPHB in eukaryotes could be similar to the functionality of polyprenols and dolichols. Thus, according to these data, PHB can theoretically be used to regulate the effect of some cytokines or growth factors on stem cells (including nonspecifically) by covalent modification of appropriate receptors by this biopolymer.

Could PHB perform some type of signaling function for microbiota bacteria when communicating with eukaryotic cells (including stem cells) of the host organism? In general, can bacteria of animal microbiota affect stem cells? Very little is known about the influence of microbiota bacteria of human and laboratory animals on the growth and differentiation of stem cells (including MSCs), but some mechanisms of these interactions have been revealed. It was shown that peptidoglycans and polysaccharides of the bacterial cell wall could alter the functional activity of MSCs. They can stimulate the immunomodulatory activity of MSCs (production of cytokines), accelerate or inhibit the growth of MSCs, and regulate the differentiation of MSCs in adipogenic, chondrogenic or osteogenic directions[104-107]. It was revealed that this biochemical response is realized through the activation of specific intracellular MSC receptors, *NOD1* and *NOD2* and receptors of the *TLR* family[104,106,108,109]. It should be noted that in cells of the immune system (for example, macrophages), activation of these receptors led to stimulation of the production of proinflammatory cytokines and an antibacterial response. It was shown that the microbiota of the oral mucosa stimulated the differentiation and development of osteoclasts by activating the regulatory genes *TLR2*, *TLR4*, *NOD1*, and *NOD2* by peptidoglycans and polysaccharides of the bacterial cell wall. This led, to bone resorption, which plays an essential role in the pathogenesis and treatment of periodontal disease[110-113]. In contrast, in MSCs, activation of these receptors leads to more complex and diverse physiological responses, such as the suppression of chronic inflammation, which can contribute to the treatment of colitis and dermatitis[108].

Some signaling substances of microbiota bacteria can simultaneously act as intermediaries in the regulation of PHB biosynthesis with bacterial communication with the host organism that deserves attention. Thus, the ability of histamine to regulate the synthesis of low-molecular-weight cPHB in *E. coli* has been demonstrated. Histamine plays an important role as a means of bacterial communication with the host organism and a regulator of the gastrointestinal tract immune system allowing bacteria to be considered "native" to the host organism. Therefore, the effect of histamine on cPHB synthesis can indicate the involvement of this biopolymer in the processes of adaptation and coexistence with the host organism[114,115]. However, it is well known that histamine is one of the most important mediators of an immune response to foreign pathogens that is produced by basophils and mast cells. Histamine is involved in the inflammatory response and has a central role as a mediator of itching (it acts as a neurotransmitter). Surprisingly, it was shown that the decrease in expression of histamine H2R receptors in human MSCs led to the suppression of cell osteogenic differentiation, which was detected by decreased expression of the osteogenic markers, osteocalcin, bone morphogenetic protein 2, and runt-related transcription factor 2[116].

Moreover, storage PHB and short-chain PHB as bacterial signal molecules can directly affect the differentiation of intestinal stem cells into mucosa enterocytes (absorptive cells). It was shown that intestinal stem cells grown in intestinal organoids *in vitro* readily uptake nanoparticles from synthetic PHAs (PLGA)[117], whereas PLGA scaffolds support their differentiation in enterocytes, goblet cells, and Paneth cells[51,52]. It should be noted that alkaline phosphatase and bone morphogenic protein are the markers of differentiation both of intestinal stem cells into enterocytes and MSCs into osteoblasts[2,4,118]. Maybe the effect of PHA scaffolds on MSC differentiation is caused by mimic action of PHAs on ALP- and BMP-pathways.

In Figure 1 we summarized the above speculations about the association of PHA natural functions and their effect on stem cells. The possible interaction of PHAs as bacteria-origin signal molecules with microbiota bacteria, intestinal stem cells, and immune cells was demonstrated in association with the effect of implanted devices from PHAs on MSCs.

The piezoelectric properties of PHB and its copolymers could also be associated with the natural functions of these biopolymers for bacteria of mammalian microbiota. There are a number of electroactive bacteria in animal microbiota, and they use electrical stimuli not only for energetic purposes but also for communication[119,120]. Moreover, some of the electroactive bacteria of mammalian microbiota, such as *Pseudomonas aeruginosa*, can synthesize and accumulate PHB and its various copolymers[121]. Considering that PHB can be covalently linked to receptors of both bacterial and eukaryotic cells[98-100], it cannot be excluded that the piezoelectric properties of the polymer can be used by microbiota both for communication among bacteria of the microbiota and with other cells of the host organism, including

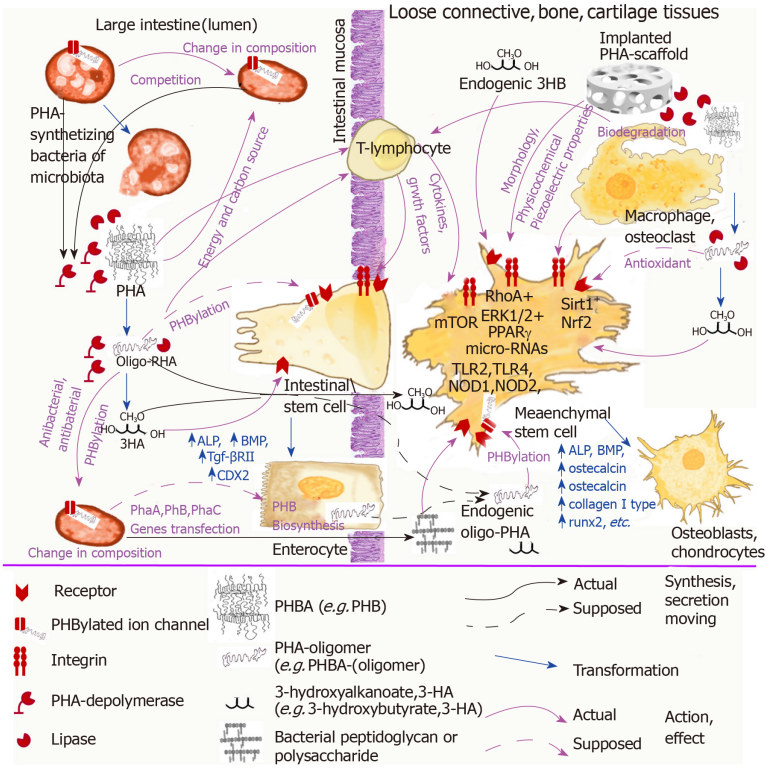

Figure 1 The scheme illustrating the association of PHA natural functions with the effect of implanted PHA scaffolds on mesenchymal stem cells differentiation. PHA: poly(3-hydroxyalkanoate), PHB: poly(3-hydroxybutyrate), 3HA: 3-hydroxyalkanoate; 3-hydroxybutyrate; ALP: alkaline phosphatase; BMP: bone morphogenetic protein; Tgf-βRII: transforming growth factor, beta receptor II; runx2: runt-related transcription factor 2; mTOR: the mammalian target of rapamycin; RhoA: Ras homolog gene family, member A; ERK1/2: extracellular signal–regulated kinases 1 and 2; PPARγ: peroxisome proliferator-activated receptor gamma; Sirt1: NAD-dependent deacetylase sirtuin-1; Nrf2: nuclear factor erythroid 2-related factor 2; TLR2 and TLR4: toll-like receptor 2 and 4; NOD1 and NOD2: nucleotide-binding oligomerization domain-containing protein 1 and 2.

intestinal stem cells.

The possibility that PHB can perform some type of signaling function in mammalian tissues can be perceptualized by the fact that the dimers and trimers of 3-hydroxybutyrate are sex pheromones in spiders[122]. Moreover, it is possible that these pheromones could also be products of the bacterial biosynthesis of the arthropod microbiota. For example, in the beetle *Costelytra zealandica*, the sex pheromone is phenol, which is produced from tyrosine in special glands by symbiotic bacteria Morganella morganii[123]. Although the dimers and trimers of 3-hydroxybutyrate were found in the fungus *Hypoxylon truncatum*, the mechanism of their synthesis was not disclosed[124].

A better understanding of the mechanisms of interaction of PHAs with MSCs is the key to developing new therapeutic agents based on them. For example, if PHB causes the osteogenic differentiation through activation of some receptor of MSCs and PHBHHx causes the chondrogenic differentiation of MSCs through activation of another receptor of MSCs, then the medical devices based on PHB should be developed specifically for bone regeneration and medical devices based on PHBHHx–specifically for cartilage regeneration. But if the osteogenic differentiation of MSCs is caused by the main product of PHAs biodegradation – 3-hydroxybutyrate, and chondrogenic differentiation of MSCs is caused by some kind of microstructure of PHBHHx medical devices, then it is better to use any type of PHA depending on their physicochemical properties (the strength and elasticity of PHB and PHBHHx differ significantly) for bone regeneration and medical devices based on any type of PHAs with desired microstructure for cartilage regeneration.

At the first case of receptor-mediated induction of MSC differentiation, it can be connected with the similar receptor-mediated action of bacteria-origin PHAs when bacteria interact with each other or with the cells of the host organism that can help to discover mechanisms of PHAs action regarding signal functions and binding with receptors of PHB oligomers. At the second case, the bone regeneration process is mediated by internal product of lipid metabolism (3-hydroxybutyrate and connected with mechanisms of its metabolism). Maybe it is better to regulate 3-hydroxybutyrate

metabolism to improve bone regeneration. The same is with the microstructure of PHAs devices because in this case, it is necessary to find the specific structural element regulating chondrogenic MSCs differentiation independent of the type of biomaterial at all. We should keep in mind that elasticity of biomaterials can also affect MSC differentiation[125].

Moreover, a more in-depth understanding of the interaction of PHAs with MSCs in relation to natural functions of these biopolymers can help to develop novel types of medical devices and pharmaceutical formulations based on them: medical devices with selective osteoinductive or chondroinductive activity and desired regenerative activity; prebiotics that promote regenerative activity of the intestinal wall through modulation of microbiota and stimulation of intestine progenitor stem cells; substrates for MSC cultivation in bioreactors; formulations for sustained delivery of drugs with biopolymer-compatible natural bioactivity; experimental model systems for MSC cultivation for drug testing; or therapeutic tissue-engineered systems simultaneously containing probiotic bacteria and MSCs as active ingredients for treatment of gastrointestinal system diseases.

CONCLUSION

PHAs have integral multifactor effects on MSCs through different mechanisms related to the main characteristics of PHA scaffolds that are difficult to separate from each other: the physicochemical properties of the polymer surface; the morphology of polymer devices; and the possible biological activity of these biopolymers. However, the intrinsic biological activity of natural PHAs plays an important role in the effect of implanted PHA devices on the proliferation and differentiation of MSCs as well as on regeneration of bone, cartilage, loose connective tissue, and other tissues.

In summary, studies that consider the problem of the influence of natural PHAs on the growth and differentiation of MSCs should be carried out in a manner that considers and integrates the functions of these biopolymers in nature. We suggest that these biopolymers of bacteria origin engage in some unexplored mechanism while signaling functions in our body through which microbiota bacteria can "communicate" with cells of the immune system, intestinal mucosa, and other tissues causing them to induce a physiological response. PHAs as bacterial signaling molecules can be involved in regenerative processes of intestinal mucosa regulated by microbiota bacteria through stimulation of intestinal stem cells differentiation into enterocytes and other mucosa epithelial cells and directly (as absorbed or endogenous oPHB) or indirectly effect the proliferation and differentiation of MSCs (Figure 1).

The above data on the participation of intercellular contact molecules such as integrins, microRNAs, the mTOR signaling pathway, antioxidant activity, and piezoelectric properties in the implementation of the biological effects of PHB and data on receptor regulation through the covalent binding of PHB to proteins can indicate the natural signaling function of this biopolymer. Unfortunately, this fascinating problem is studied very poorly. There is only very fragmentary and incomplete information. A broader understanding of the role of PHAs in nature could help to further clarify the unusual or contradictory data in the field of the interactions of PHAs with MSCs and to develop novel medical devices and pharmaceutical formulations based on them.

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REVIEW

Aging: A cell source limiting factor in tissue engineering

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Abstract

Tissue engineering has yet to reach its ideal goal, *i.e.* creating profitable off-the-shelf tissues and organs, designing scaffolds and three-dimensional tissue architectures that can maintain the blood supply, proper biomaterial selection, and identifying the most efficient cell source for use in cell therapy and tissue engineering. These are still the major challenges in this field. Regarding the identification of the most appropriate cell source, aging as a factor that affects both somatic and stem cells and limits their function and applications is a preventable and, at least to some extents, a reversible phenomenon. Here, we reviewed different stem cell types, namely embryonic stem cells, adult stem cells, induced pluripotent stem cells, and genetically modified stem cells, as well as their sources, *i.e.* autologous, allogeneic, and xenogeneic sources. Afterward, we approached aging by discussing the functional decline of aged stem cells and different intrinsic and extrinsic factors that are involved in stem cell aging including replicative senescence and Hayflick limit, autophagy, epigenetic changes, miRNAs, mTOR and AMPK pathways, and the role of mitochondria in stem cell senescence. Finally, various interventions for rejuvenation and geroprotection of stem cells are discussed. These interventions can be applied in cell therapy and tissue engineering methods to conquer aging as a limiting factor, both in original cell source and in the *in vitro* proliferated cells.

Key words: Aging; Senescence; Rejuvenation; Geroprotection; Tissue engineering; Stem cell therapy

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Core tip: To attain profitable off-the-shelf tissues and organs, we must deal with the challenge of identifying and isolating an optimal cell source. Different types of stem cells with different properties have been used in tissue engineering and cell therapy to face this challenge. Although aging is an inevitable process that can eventually limit the function and stemness of stem cells, it is a conquerable phenomenon. In this article, we have reviewed several applicable interventions that can be used to overcome cellular aging.

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INTRODUCTION

Anatomical and functional complexities of biological systems challenge the artificial construction of viable human tissues and organs. Proper three-dimensional tissue architecture to maintain blood supply is a key constraint on the size of the *in vitro* fabricated tissues[1]. In addition, biomaterial selection and strategies to design tissue scaffolds are vital for regulating cell signaling pathways that provide appropriate cell-cell interactions such as growth factor delivery, which is essential for cell differentiation. Although numerous attempts were made to overcome these key challenges in tissue engineering, the reproducible *in vitro* construction of artificial vascularized tissue is still needed[2].

The ideal goal of tissue engineering is to create off-the-shelf tissues and organs providing vast opportunities to tackle a group of diseases and to reduce the need for organ donors. This not only would treat millions of patients, but also increase human longevity and quality of life[1,3,4]. As the field of tissue engineering evolves, new obstacles appear in the way of the research and clinical application of these artificial tissues and organs. The fundamentals of this interdisciplinary field not only involves identifying biomaterials and designing scaffolds for *in vivo* cell expansion but also requires addressing the reliable cell sources. Hence, gradual advances in the clinical application of tissue engineering deal with hurdles in diverse aspects of science such as cell biology, bioengineering, and material science[5].

Apart from these engineering challenges, biologic issues and the major concern of identifying the ideal cell source is the other essential principle of tissue engineering[2]. Various stem cell types and sources have been extensively employed in regenerative medicine studies. However, each source has its own practical and technical challenges concerning their availability, isolation and cell expansion, cell delivery, aging, immunological barriers, and clinical and therapeutic efficiency. Furthermore, while major challenges of tissue engineering must be addressed at first, aging, as a cell source limiting factor, should not be overlooked. In this article, we have reviewed the cell sources that are used in tissue engineering and cell therapy techniques and how aging and cell senescence can challenge the isolation of ideal cell source. Also, we have discussed potentially applicable approaches for rejuvenation of aged cells.

CELL SOURCE AS A MAJOR CHALLENGE

First and foremost, the unresolved controversy of identifying the optimal cell types for tissue engineering is still a major challenge[4,6,7]. While cell transplantation, organ transplantation, and tissue engineering are fundamentally different, there are essentially three varieties of sources: Autologous, allogeneic, and xenogeneic cells, each of which can be subdivided into several types of stem cells including adult and embryonic stem cells. In addition, the discovery of induced pluripotent stem cells (iPSCs), which are discussed in the following sections, represent a promising source of cells for all branches of regenerative medicine[8,9].

Autologous sources

In autologous transplantation, the donor and the recipient are the same. Concerning the role of the immune system in potential tissue rejections, utilizing a patient's own

cells or "autologous cells" would be ideal. This method minimizes the chance of graft *versus* host disease and transmitted infections, and more importantly it would eliminate the need for lifetime use of immunosuppressive drugs, which improves the quality of life in post-transplant patients[10]. Despite these benefits, autologous cell therapy brings about several challenges. In fact, using the patient's own cells might not be practical for the majority of cases. Transplant waiting lists are filled with aged patients who suffer from age-associated morbidities and cellular senescence affecting both their somatic and stem cells[11]. In addition, the patients who suffer from gene defects cannot easily benefit from autologous cell therapy[12]. Furthermore, to be viable for tissue engineering, millions of autologous cells should be collected from a donor and expanded *ex vivo*. For many tissue types, harvesting a sufficient number of cells is not applicable, especially when a patient is aged or severely diseased. Moreover, cell culture *per se* can cause undefined complications; the proliferative potential and clonogenicity of stem cells decrease after several cell divisions, which raises concerns about viability and functionality of cells after transplantation. These issues make autologous cell therapy undesirable for clinical applications, especially in emergencies or acute phases of disease[9,13].

Allogeneic sources

As mentioned earlier, the goal of tissue engineering is to manufacture large quantities of off-the-shelf tissues and organs that are immediately available to be administered clinically[14]. Allogeneic cells are cells from a genetically non-identical donor but of the same species. Thus, unaffected cells, tissues, and organs of every healthy donor can be a precious allogeneic cell source. This will rule out the challenges of aging, unavailability, and *in vitro* expansion challenges of autologous cell sources and consequently introduce allogeneic cell therapy as a promising method in case of emergency. This advantageousness paved the way for preparing a master bank of ready-made, clinically practical, and off-the-shelf allogeneic cells. On the contrary, the immunogenicity of allogeneic cells and the major histocompatibility complex (commonly known as MHC) incompatibilities are by far the most formidable barriers of allotransplantation. In addition, the side effects of immunosuppression like metabolic disorders, malignancies, and opportunistic infections can aggravate the outcome of a transplantation[9,12,15].

Xenogeneic sources

Xenogeneic or cross-species transplantation is the process of transplanting living cells, tissues, or organs from one species to another. In recent decades, the ever increasing demand for clinical transplantation and shortage of allogeneic sources for patients on the waiting lists has led to considerable amounts of clinical and experimental research in order to overcome the barriers of xenotransplantation. However, a great number of ethical red tape and immunological roadblocks are yet to be surpassed. Graft rejection and failure to achieve successful long-term outcomes are the main issues to be addressed, as there are great disparities between MHCs of different species. Another concern is the risk of zoonotic infections, particularly unidentified viruses. In addition, xenotransplantation is by itself a controversial ethical issue and sometimes raises religious concerns because it involves sacrificing animals to harvest organs and tissues for human usage[16].

CELL TYPES

Thus far, several stem cell types have been utilized in the field of tissue engineering.

Embryonic stem cells (ESCs)

ESCs are pluripotent stem cells isolated from the inner cell mass of blastocysts up until day 5.5 post-fertilization, right before the stage in which the embryo is ready for gastrulation[17,18]. They have unlimited potential for self-renewal and differentiation to be used as a source for derivation of multiple lineages of adult cells. In spite of these distinctive potentials, studies have raised concerns over the prolonged culture of ESCs. Formation of *in vivo* teratomas has been reported in implantation of *in vitro* cultured undifferentiated ESCs. Additionally, difficulties in finding patient-matched ESCs are an obstacle. Finally, because isolating ESCs involves the destruction or manipulation of pre-implantation stage embryo, there are lots of ethical controversies surrounding their usage[19].

iPSCs

iPSCs are generated *via* the induction of expression of certain genes in non-pluripotent adult cells. This technique was first developed in 2006 by Takahashi and

Yamanaka, who introduced four transcription factors, Oct-4, c-Myc, KLF4, and SOX2, into mouse fibroblasts. These factors contributed to the maintenance of pluripotency in ESCs and are sufficient to generate ESC-like colonies[20]. A year later, Yamanaka improved on the reprogramming approach, leading to generation of iPSCs that were indistinguishable from ESCs[21]. Direct derivation of iPSCs from adult tissues not only helps to bypass the need for embryos as the pluripotent stem cell source but also makes personalized cell therapy a viable option. This method could generate unlimited supplies of young autologous pluripotent stem cells with a promising future in the field of regenerative medicine[22,23].

In contrast, several challenges still exist. Primarily, the efficacy of the reprogramming process is considerably low. For example, the rate at which somatic cells were reprogrammed into iPSCs in Yamanaka's original mouse study was 0.01%–0.1%. Although protocols for the induction of pluripotency are evolving, experimental evidence for appropriate initial cell type, transcription factor combinations, gene vectors, and methods of cell culture still lack the consistency required for clinical applications. In addition, induction of pluripotency and the process of reprogramming, itself causes genomic instability and adversely affects the cellular integrity. Moreover, the reprogramming factors (such as c-Myc) are known to be proto-oncogenes. Also, the retained epigenetic memory of the past somatic identity in newly generated iPSCs may influence the potency and *in vivo* functionality of engineered tissues[24]. However, recent rapid progress of several clinical studies have improved the outlook for this technology[25-27]. The first iPSC-derived therapy was done for age-related macular degeneration patients at Japan's RIKEN Institute[28]. Encouraging results have smoothed the path for other scientific groups to seek clinical trials for the iPSC-based treatment of cardiac diseases, Parkinson's disease, and blood clotting disorders[29-32].

Adult/somatic stem cells

Adult stem cells are populations of undifferentiated cells that unlike ESCs are found in mature tissues and organs throughout the postnatal life. These progenitor cells are responsible for tissue cell turnover and maintenance of injured tissues. Their easy accessibility, availability, and self-renewal ability introduce adult stem cells as a preferred cell source for transplantation. In spite of these great potentials, adult stem cells are not perfect. First, unlike pluripotent ESCs, adult stem cells are usually multipotent and can only give rise to a limited number of cell lineages of their specific tissue. Although adult stem cells can be obtained from both allogeneic and autologous sources, the age-dependent progressive deterioration of stem cell function is an important issue to be expected[11,33]. In addition, due to replicative senescence after prolonged culture periods, the proliferative ability of these cells declines rapidly[9,33].

Genetically-modified stem cells

Genetically modified stem cells are born out of the junction of two focus points of intense research: Gene therapy and stem cell therapy. Gene modification of cells prior to transplantation is one of the proposed solutions to overcome cell source challenges and to enhance cell proliferation and function[34]. Various gene therapy approaches are proposed, including the creation of genetically or epigenetically modified cells expressing useful proteins, growth factors or growth factor receptors, transcription factors, neurotransmitters and their receptors, and neuropeptides or creating cells that have the ability to recruit host cells to the implantation site[35,36].

The promise of using clustered regularly interspaced short palindromic repeats (CRISPR) technology brings about new hope as a tool for the gene editing of stem cells[37,38]. Brunger *et al*[39] used CRISPR for targeted deletion of the interleukin-1 receptor type 1 gene in murine iPSCs to make custom-made inflammation resistant cartilage cells. Genome editing by CRISPR has also been used to correct Duchenne muscular dystrophy patients derived iPSCs successfully to differentiate muscle cells that express functional protein[40]. Moreover, genetic modification of patient-derived iPSCs using CRISPR and other genetic engineering tools has been used for hemoglobinopathies such as β-thalassemia and sickle-cell anemia[41-44].

Immortalized cell lines are genetically mutated cells with unlimited propagation potential that are generated to prevail major challenges of cell source availability, such as early onset of cellular senescence and the consecutive limited cell expansion and differentiation potential. The mutations required for immortality can occur naturally or can be induced intentionally. There are several possible gene modification methods to bypass the senescence block. Viral oncogenes such as SV40 and E6/E7 proteins of oncogenic human papillomaviruses are used for regulating human cellular senescence[45-48]. One possible gene editing approach is artificial expression of key proteins required for immortality such as telomerase (discussed in later parts of this article). However, it was reported that they might be associated with genomic

instability and increased risk of cell transformation[33,49,50].

Conditionally reprogrammed cells are another gene therapy approach used to rapidly and efficiently generate an unlimited number of patient-derived cells. In this technique by using both fibroblast feeder cells and a Rho-associated kinase inhibitor, Liu *et al*[51] indefinitely extend the life span of primary human keratinocytes *ex vivo*. Unlimited propagation of these karyotype-stable and non-tumorigenic cells offer opportunities for regenerative medicine as these cells have a stem cell-like phenotype[52].

In spite of preliminary success, several hurdles prevent both laboratory and particularly clinical applications of these gene-editing technologies. Genetic and epigenetic changes might cause unresolved issues to the patient. Transgenic genes, vector genes, or non-autologous stem cells might trigger immune reactions or even induce neoplastic transformation. In addition, developing an ideal gene vector system is next to impossible; the most common vectors are viruses. Beyond the uncontrollable insertional mutagenesis of viruses leading to increased risk of malignant transformation, viruses can cause adverse events such as toxicity and immune and inflammatory responses[34,53].

Ethical issues of gene editing of stem cells should not be overlooked. Matters like safety and efficacy of gene editing, including off-target mutations, raise concerns regarding human enhancement and eugenics that must be closely regulated. All in all, it is a necessity to set boundaries for techniques that have dire consequences[37,54].

CELLULAR AGING AS A LIMITING FACTOR

Aging, whether it is in a stem cell or a fully differentiated cell, seems to be the result of particularly shared processes. Some believe that aging occurs due to the incapability of senescent stem cells to contribute in tissue repair and regeneration, while others suggest that the vicious cycle of the dysfunctional relationship between stem cells and their niche cells is the leading contributor in the progressive deterioration during aging[55].

Cell intrinsic changes usually occur due to the accumulation of damage caused by normal cellular processes like metabolism and proliferation, while cell extrinsic changes are derived by a factor external to the cell subjected to those changes, such as paracrine and endocrine factors, ionizing radiation. and changes in the extracellular matrix[55]. Sometimes it is almost impossible to delineate the intrinsic and extrinsic changes. For instance, free radicals produced during both oxidative phosphorylation (a cell intrinsic factor) and generated by ionizing radiation (a cell extrinsic factor) can harm cellular components leading to senescence[56]. These intrinsic and extrinsic elements are discussed in detail in the following sections.

Functional decline of aged stem cells

During aging, several functional properties of the stem cells are being affected[55]. For instance, aged stem cells, especially neural stem cells[57-59], germline stem cells[60], and muscle satellite cells[61-65], lose their cellular polarity. As a consequence, they lose their ability to divide asymmetrically, a key feature of stem cells helping them to preserve the stem cell repertoires[55]. This loss of polarity is granted mostly by cell extrinsic factors like aged niche cells, dysfunctional adhesion molecules, disrupted morphogen, growth factors signaling, and inflammation[66-71]. Another phenomenon that is seen in aged stem cells is a lineage bias in the differentiation of their progenies. To enumerate, aged hematopoietic stem cells (HSCs) tend to skew toward the myeloid lineages more prominently compared to young and fully functional HSCs, a circumstance that leads to the incompetence of adaptive immune system in aged individuals[55,72-83].

Another example is loss of osteogenic differentiation and biased adipogenic commitment of mesenchymal stem cells (MSCs), which contributes to osteoporosis and bone marrow fat accumulation in aged individuals. Over-expression of receptor activator of nuclear factor kappa B ligand, down-regulation of peroxisome proliferator-activator receptor gamma, and suppression of forkhead box family O3 by protein kinase B (AKT) signaling in aged MSCs are proposed as the mechanisms responsible in this phenomenon[84-86]. This age-associated skewed differentiation is not completely understood for cells like ESCs and iPSCs. For instance, Xie *et al*[87] showed that H9 ESCs have an increased tendency for ectodermal lineages; however, this may be explained by the culture media composition. However, they observed no difference in teratoma formation between old and young ESCs. iPSCs were found to have a different story; while some studies have claimed that iPSCs have skewed differentiation capacity, probably because of their retained epigenetic memory of their original cell lines[88], other studies have reported that the iPSC differentiation capacity

has no correlation with the cell source they are originated from[24,89].

Additionally, aged stem cells lose their migratory and homing potential, due to both cell intrinsic and cell extrinsic changes. For instance, transplantation of young mouse HSCs to old individuals delivers a lower yield compared to young recipients, which is due to the inferiority of the aged bone marrow niche[90,91]. Additionally, transplantation of old HSCs to young individuals is less effective in contrast to young donor cells, which shows a decline in the homing capacity of old stem cells due to intrinsic factors[72,90,91]. Another interesting aspect of HSCs is that using immunophenotyping, it has been shown that the number of HSCs increases with age; however, functional evaluation of these immunophenotypes shows reduced engraftment and improper differentiation in the new host[55,78-80,92]. This indicates a decrease in the population of functional HSCs or a form of clonality that happens with aging[55,81-83].

Yet, another example is heterochronic transplantation of aged mice muscle satellite cells into young recipients[61-65]. These cells show much lower regenerative capacity in old donors compared to young recipients, mostly due to cell extrinsic factors including, but not limited to, Wnt, Notch, and transforming growth factor beta signaling as well as altered Janus kinase-signal transducer and activator of transcription signal transduction[93,94]. In addition to the declined *in vivo* engraftment potential, aged satellite cells also have a reduced *in vitro* proliferation capacity[95-99].

Replicative senescence, Hayflick limit, and telomere length

Replicative senescence, equally known as Hayflick limit, is a phenomenon observed *in vitro* in which a primary cell or a stem cell stops dividing after a particular number of doublings. While the mechanism of Hayflick limit is not thoroughly understood, many attribute telomere attrition and genomic instability as the principal mechanism of replicative senescence[84,100]. For instance, MSCs stop dividing after 20 to 40 doublings when their telomeres are between 5.8 and 10.5 kb[84,101-103]. Despite the fact that telomere attrition can be considered an intrinsic change in stem cell replicative senescence, several questions remain to be addressed. Considering the fact that non-dividing cells also senesce[104-106], can we really take telomere attrition as one of the integral causes of aging, or is it just one of the many "effects" of the aging process that worsens this vicious cycle? Additionally, if telomere attrition is a deriving cause for aging, is it possible to increase the lifespan of a model organism, like a mouse or a rat, by "telomerization" or telomere lengthening? While the answers to these questions are controversial, it seems possible to immortalize cell lines *via* expression of telomerase subunits. Human telomerase reverse transcriptase (hTERT)-immortalized cells show extended life span with improved functional activities[58]. A successful example of this approach is the use of immortalized human keratinocyte cell lines in the treatment of chronic wounds and complex skin defects[59,60]. Notwithstanding, one study showed that over-expression of TERT only increases the median lifespan of the cancer-resistant mice, implying that telomere attrition might be important only in the late stages of aging[55,107,108]. Additionally, as mice have very long telomeres, it is not clear why they have a much shorter lifespan. Knocking out the RNA component of telomerase has no obvious life-threatening effect up to the sixth generation of these mice. Albeit, HSCs of the fourth generation started to show lineage skewness[109,110].

Stem cells spend most of their life in a quiescent state, probably to avoid the replicative damages, especially those related to DNA duplication. These quiescent stem cells are more likely to acquire destructive DNA damage after a double-strand break compared to a cell in its proliferative state. Quiescent stem cells mostly use the error-prone non-homologous end joining repair mechanism, while proliferative cells utilize homologous recombination, a much more accurate repair mechanism[111,112].

Autophagy

Perhaps one of the most extensively studied factors involved in aging is autophagy. Autophagy is a conserved mechanism that has evolved to recycle the damaged structures and organelles in a eukaryotic cell. This very sophisticated feature integrates the signal from several pathways to regulate the level of protein degradation. AMPK (adenosine monophosphate-dependent protein kinase), mTOR (mechanistic target of rapamycin), and ULK1 (Unc-51 like autophagy activating kinase 1) are the most important upstream signaling pathways of autophagy that regulate atg (autophagy related) genes and autophagosome formation. AMPK senses the ratio of AMP:ATP and activates ULK1 whenever the cell requires more energy. mTOR, on the other hand, inhibits ULK1 and autophagosome formation whenever it integrates the signal from nutrients and growth factors, which are the prerequisites of anabolism[11,113,114]. Every cell tries to strike a balance between the three forms of autophagy (macro-autophagy, micro-autophagy, and chaperone-mediated autophagy) and protein synthesis[115]. It has been shown that autophagy is decreased

in aged cells of both animal models and humans, regardless of whether it is a stem cell or a fully differentiated one. While autophagy declines progressively, tiny amounts of damage gradually accumulate throughout time[115-117]. Stem cells have at least two mechanisms to prevent these damaged proteins and organelles to build-up: the asymmetric division (which diminishes with aging)[55,118-120] and maintaining high levels of autophagy and proteasome activity[55,121].

Aged MSCs and HSCs show accumulation of autophagic vesicles and inclusion bodies with LC3II or ubiquitin expression, which are the features of decreased autophagy with age. Rapamycin or spermidine treatment restores the autophagic capacity, leading to clearance of those accumulated autophagic vesicles and inclusions[115,122,123]. Additionally, Ho *et al*[123] showed that more than two-thirds of the HSCs in an aging population have very low levels of autophagy and skewed and escalated differentiation to myeloid lineages, while only less than a third of them had high levels of autophagy and regenerative potential comparable to that of young HSCs[115,123]. It has also been shown that while young MSCs in GFP-LC3 transgenic mice have high levels of autophagy in the quiescent state, this capacity fades with aging[115]. Furthermore, using conditional knock-out mice, when autophagosome formation is genetically compromised, senescence can rise from impairment of proteostasis[115,122]. These studies substantiate the importance of autophagy for maintaining stemness in a quiescent stem cell[115].

Epigenetic changes

In a fertilized egg, the genome of two mature and non-young individuals get stripped of epigenetic marks (except for the imprinted areas) to form a new very young individual with almost the same lifespan as the parents[124]. This epigenetic reprogramming can be emulated by *in vitro* induction of totipotency/pluripotency. The nucleus of a somatic cell can either be fused with the cytoplasm of an enucleated oocyte (somatic cell nuclear transfer) or be transfected with viruses expressing Yamanaka factors (SOX2, c-Myc, Oct-4, and KLF4)[20] to produce an iPSC[55,125-128]. Although this reprogrammed cell is very similar to a freshly young ESC in many aspects, its epigenome is slightly different[127]. In fact, iPSCs are reprogrammed with regard to the age-, tissue-, and senescence-associated DNA methylation patterns but keep some donor-specific DNA methylation patterns[129]. In 2013, Abad *et al*[130] produced a transgenic mouse expressing the four Yamanaka factors in every cell upon administration of doxycycline. These mice usually develop teratomas in several organs and tissues. Another interesting fact about epigenetic reprograming is that it is possible to make phenotypically young neural stem cells from iPSCs, which are generated from aged fibroblast, while direct transdifferentiating neural stem cells from aged fibroblasts maintains the aged phenotype[55,131].

This reprogramability of the epigenome helps us to unravel detailed mechanisms of aging in order to find a way to conquer it in the future; however, it is obviously not a practical formula for rejuvenation[55]. Perhaps it is better to use epigenomic results to reinforce gene regulatory networks and to decipher what signals are differentially active in old cells compared to their younger counterparts.

miRNA

Among the differentially expressed genes in aged cells in comparison with the young cells, there are several non-coding RNAs, including miRNAs expressed (some of which are even proposed as biomarkers for aging)[56,84,132]. miR-195 is over-expressed in aged cells and reduces the telomerase reverse transcriptase; also, knocking-down this miRNA in MSCs increases their regenerative capacity when transplanted to the infarcted myocardium[56,133]. Another example is over-expressed miR-34a, which is elevated in infarcted mouse hearts and is associated with apoptosis and senescence. Also, its inhibition decreases the number of apoptotic cells in cardiac tissue[56,134,135]. Some of these differentially expressed miRNAs control proliferative and regenerative capacity of the stem cells by regulating cell cycle transition and stemness factors (such as Nanog)[84,133,136-138].

Role of mitochondria in stem cell senescence

Free radicals, otherwise known as reactive oxygen species (ROS), are a well-recognized origin of age-related molecular injuries including but not limited to nuclear and mitochondrial DNA mutations, organelle damages, and lipofuscins. Chronic inflammation, ionizing radiation, and mitochondrial dysfunction are the most prominent sources of ROS in cells[56,139,140]. Stem cells employ several mechanisms to keep ROS and its damage at bay. To enumerate, quiescent HSCs depend predominantly on glycolysis to limit ROS production[75,141].

Sirtuins, (SIRT1–SIRT7) a conserved family of NAD^+-dependent deacetylases of which SIRT1 is the best known, appears to increase mitochondrial turnover by

activation of mitophagy. Activation of SIRTs can considerably extend the replicative capacity of human bone marrow stem cells[142] and human fibroblasts[11,143]. Additionally, SIRTs boost the stress-relieving and antioxidant mechanisms in cells. Studies show that over-expression of certain SIRTs increases catalase and superoxide dismutase, while their knock-down compromises cell proliferation and increases cellular senescence[56,144-147].

On the other hand, stem cells have a unique way to get rid of the damaged proteins and organelles[55,75]. During asymmetrical division, stem cells actively accumulate these injuries in the differentiating daughter cell, while keeping the daughter stem cell almost clean of them[109,118-120,148]. This polarized division is lost in certain stem cells, like HSCs and germline stem cells, during aging. As a matter of fact, less-polarized HSCs are more biased toward myeloid lineages[55,70,71]. Furthermore, autophagy and proteasome-mediated degradation, the other mechanisms that keep even the quiescent stem cells clean, diminish with aging. When dysfunctional mitochondria cannot be recycled by mitophagy (macro-autophagy of mitochondria), it generates more ROS. As a result, we observe a vicious cycle between impaired autophagy, mitochondrial dysfunction, and ROS-mediated injuries[56,139,140,149]. Xie *et al*[87] found that the most prominent changes that occur in long-term passaged ESCs have to do with the mitochondria; older passages of H9 and PKU1 hESC lines have elevated mitochondrial mass, ROS level, and mitochondrial membrane potential. On the other hand, aged iPSCs develop defects in their nuclear envelop[150], which might be the cause of interference in SIRT and NF-kB nuclear transportation and downstream signaling in these cells[151,152].

Above all, some studies contradict ROS as a contributing factor in aging. At the cellular level, Zhu *et al*[153] showed that there is "no evident dose-response effect between cellular ROS level and its cytotoxicity." For instance, they showed that while all three of the piperlongumin, beta-phenylethyl isothiocyanate, and lactic acid increased ROS in the cultured cells, only piperlongumin and beta-phenylethyl isothiocyanate, two ROS-based chemotherapeutic agents, killed the cells and lactic acid "spared them." Additionally, although chemical depletion of glutathione increased ROS much higher than piperlongumin and beta-phenylethyl isothiocyanate, it did not affect the cell growth in cultured samples. However, these results were achieved in cancer cells, and it is unclear if similar mechanisms also happen in stem cells. Le Gal *et al*[154] showed that administration of the antioxidant N-acetyl cysteine to a mouse model of melanoma not only decreased the survival of the mice but also increased the severity of their tumors by increasing metastasis. Biesalski *et al*[155] meta-analytically reevaluated clinical effectiveness of antioxidants on mortality and health. They showed that micronutrients, including those with antioxidant activity, are only effective in those with the deficiencies or the risk of deficiencies, but not effective in individuals with the micronutrients above the minimum required level. All in all, these counterexamples provide sufficient evidence to raise a reasonable doubt toward ROS-based therapeutics.

INTERVENTIONS FOR REJUVENATION OF AGED STEM CELLS

The ultimate goal of unraveling mechanisms of aging is geroprotection (preventing from aging) or rejuvenation (making a senescent cell young again). For this purpose, there are three options: Changing the extrinsic factors, altering the intrinsic factors, or manipulating the genomic targets of those changes. We can either use pharmacological means, modify the environment in which the stem cells reside, or genetically manipulate the stem cells.

Using pharmacological means to prevent aging or even rejuvenate is, perhaps, the most practical measure. Different mechanisms have been targeted pharmacologically. For instance, antioxidants like vitamin C and N-acetyl cysteine have been used to reduce ROS both *in vitro* and *in vivo*[75,154,156]. However, their efficacy is limited, especially *in vivo*. Although antioxidants to some extent show geroprotection in cell culture, the possible life extension by reduction of free radicals is challenged by *in vivo* experiments[115,153-155]. Comparatively, SIRTs are another example of drug targets for geroprotection. Resveratrol, resveratrol-mimicking compounds, and NAD+ seem to hinder aging both *in vitro* and *in vivo* by activating certain members of the SIRT family[157-160]. In particular, resveratrol improves metabolism and enhances DNA repair, which are critically important in aging[11,157].

Metabolic dysfunction is yet another focus for research on geroprotection. Rapamycin, spermidine, quercetin, and metformin are only a few examples of the drugs that increase the lifespan by this mechanism, whether it is in cell culture or *in*

vivo[56,161]. As we previously discussed, the balance between protein synthesis and protein recycling is disrupted in aging. Rapamycin that inhibits mTOR gives an advantage to the autophagic side of the balance between autophagy and protein synthesis[56,114,162]. Likewise, metformin activates AMPK to increase autophagy and other anti-stress mechanisms in the cell and slightly inhibits mTOR complex 1 through targets upstream of mTOR complex 1[11,163,164]. This boosted autophagy helps the cell to get rid of the damaged organelles and macromolecules much faster than it did before. Thus, it delays the damage accumulation, which is an important factor in dysfunctioning of stem cells[73,115,165-167]. Both of these small molecules have been shown to increase the lifespan, decrease the doubling time, and improve functional properties of stem cells, *e.g.*, engraftment, migratory, and regenerative potential[11,115,165-167]. Furthermore, combination treatment of cardiac stem cells with rapamycin and resveratrol improves the cardiac output of the infarcted myocardium in mice[56,168].

Caloric restriction (CR) is the most effective intervention for lifespan extension. Mechanistically, CR exerts its benefits through the alteration of the nutrient/growth factor-sensing mTOR signaling, energy-sensing AMPK signaling, stress-fighting forkhead box family O signaling, and SIRTs[55,169-172]. Therefore, CR not only increases the longevity of stem cells but also enhances the performance of niche cells that support stem cells[55,173-176]. Interestingly, most of the compounds that extend the lifespan by improving metabolism, especially those that promote autophagy like metformin and spermidine, are known as CR mimetics[73,174,177,178]. In fact, the geroprotective activity of CR is repressed by hindering autophagy[73,117]. While CR works best *in vivo*, it is not a practical way to extend the *in vitro* lifespan of stem cells, precisely because it limits the doubling time of cells. Thus, these CR mimetics might be the most practical intervention to be used in cell culture[11].

Although interventions like genetic manipulation might effectively work to counteract senescence in stem cells, their cost and safety concerns limit their application[75,179]. Studies mentioned interventions like over-expression of telomerase as a proposed mechanism for counteracting replicative senescence in MSCs[84,180]. For instance, over-expression of hTERT in MSCs increased their lifespan, while the normal karyotype was maintained[84,180,181]. Another strategy to genetically prevent aging is knocking-down either the retinoblastoma protein gene or the $p16^{INK4a}$ gene[84,182,183]. Retinoblastoma gene silencing decreases the age-related DNA damage and senescence as well as increases the functionality of MSCs[84,182]. Finally, manipulating miRNAs could be an effective strategy, but it needs further experimental support. To enumerate, knock-down of miR-195 leads to increased expression of hTERT, and forkhead box family O3 also intensified phosphorylation of protein kinase B (AKT) in senescent MSCs[84,133].

CONCLUSION

The way toward the production of tissue engineered products still has serious hurdles to overcome: The choice of cell source, proper biomaterial selection, maintaining blood supply by designing suitable scaffolds, and three-dimensional tissue architecture. Combined efforts to prevail over these major obstacles are warranted to pave the way for achieving tissue engineered products at a commercial scale.

With regards to the choice of cell source, aging is a limiting factor. Aging, as inevitable as it seems, is proven to be conquerable. In different cell types the problem of aging is preventable and to some extent reversible. As aging is a very complex and dynamic phenomenon, it would be better to approach it from a systems biology point of view to reach the best results. Perhaps we need to target multiple pathways to find the maximum efficacy. Regardless of the application of the stem cells, *i.e.* tissue engineering and cell therapy, we have to overcome aging, both in the original cell source and in the *in vitro* proliferation.

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REVIEW

Microfluidic three-dimensional cell culture of stem cells for high-throughput analysis

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Abstract

Although the recent advances in stem cell engineering have gained a great deal of attention due to their high potential in clinical research, the applicability of stem cells for preclinical screening in the drug discovery process is still challenging due to difficulties in controlling the stem cell microenvironment and the limited availability of high-throughput systems. Recently, researchers have been actively developing and evaluating three-dimensional (3D) cell culture-based platforms using microfluidic technologies, such as organ-on-a-chip and organoid-on-a-chip platforms, and they have achieved promising breakthroughs in stem cell engineering. In this review, we start with a comprehensive discussion on the importance of microfluidic 3D cell culture techniques in stem cell research and their technical strategies in the field of drug discovery. In a subsequent section, we discuss microfluidic 3D cell culture techniques for high-throughput analysis for use in stem cell research. In addition, some potential and practical applications of organ-on-a-chip or organoid-on-a-chip platforms using stem cells as drug screening and disease models are highlighted.

Key words: Stem cell; Microfluidic technology; Three-dimensional cell culture; High-throughput screening

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Core tip: A recent advance of microfluidic techniques using stem cells for high-throughput assay is described. Induced pluripotent stem cells and the innovative organ-on-a-chip or organoid-on-a-chip have led to progress in *in vitro* drug screening platforms. We summarized the various examples of microfluidic techniques, including organ-on-a-chip or organoid-on-a-chip using stem cells for high-throughput screening, and discussed the current challenges and future perspectives of microfluidic technologies in stem cell research.

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INTRODUCTION

Stem cell engineering, the interface of engineering with the world of stem cells, has emerged over the last decade and covers fields from the basic science to engineered approaches[1]. With the significant advances in the development of stem cells technologies, many approaches have been introduced for modeling genetic diseases, and these models have been made available for applications, such as *in vitro* drug tests[2-5]. Usually, immortalized cell lines lack the differentiated functions of specific organs, and they may not display the disease-specific or patient-specific phenotypes. Also, these cell lines may include oncogenic factors, such as SV40, during the transformation[6]. Stem cells self-renew extensively and have pluripotency in that they can differentiate into all types of cells in an organism. Thus, stem cells have gained significant attention in providing a variety of specialized cells that are relevant for modeling human development and disease as well as applications in regenerative medicine[7-9]. However, stem cells tend to be very sensitive to various biochemical and physiological cures, and their fate is altered easily by their microenvironment. Also, stem cells themselves cannot recapitulate the microenvironment that is physiologically relevant to the complex structure of human organs.

Recently, emphasis has been placed on the roles of the three-dimensional (3D) cell culture techniques that can precisely control multiple cues in the biological microenvironment of stem cells. The 3D cell culture systems are comprised of organ-specific cells and their microenvironments, so they were able to mimic human physiology more accurately. Indeed, organ-on-a-chip platforms consist of tissue-specific cells and their extracellular matrixes (ECMs) that can remodel 3D tissue architectures and also mimic the physiological conditions, such as shear stress and fluidic flow[10,11]. In this regard, microfluidic devices are ideally suited for stem cell cultures and their maintenance by providing a way to recreate a microenvironment *in vivo*. Also, this system has flexibility and feasibility that can be coupled to robust hardware systems that are capable of high-throughput analysis, rapid sampling, and liquid handling, allowing them to process hundreds of samples[12,13]. Such advantages have led to the innovative development of organ-on-a-chip or organoid-on-a chip systems based on stem cells and their applications in high-throughput drug screening[9,14-16].

In this review, we discuss the most recent advances in 3D microfluidic technology in the field of stem cell research and their applications for high-throughput screening (HTS). Also, we review the progress that has been made to generate organ-on-a-chip platforms and, more recently, organoid-on-a-chip, particularly with an emphasis on important innovations of different microfluidic aspects to improve stem cell research for high-throughput analysis. Then, we discuss how these technologies combined with high-throughput analysis might be enhanced in the future.

3D MICROFLUIDIC CELL CULTURE

It is difficult to maintain the cellular functions in conventional two-dimensional (2D) cell cultures for prolonged periods of time because these cultures lack the physiological microenvironment of *in vivo* tissue. Such cell systems may not be able to prove the real cellular response to drugs due to their inability to control and mimic the microenvironment of complicated organs. Also, drug diffusion kinetics is not

modeled accurately in a 2D cell culture. Therefore, 2D cell cultures increase the chances of providing misleading and non-predictive preclinical results for *in vivo* test[17,18]. On the other hand, *in vivo* animal tests have traditionally been the gold standard models for preclinical efficacy tests in the drug discovery process, but various issues still exist, such as ethical issues and genetic differences between species. In addition, animal models have many drawbacks, such as high cost and uncertainties in the interpretation of the results in many pathological studies. Due to these weaknesses of the traditional models, an alternative cell culture model that corresponds to an *in vivo* system is required in order to obtain better predictions of the preclinical response to drugs.

In recent years, advances in microfluidic technology in 3D cell cultures have resulted in promising alternative methods to the conventional *in vivo* and *in vitro* models in the field of drug development[4,15,19-23]. In nature, the fate of *in vivo* cells is affected largely by external physical and chemical factors, and cell-cell and cell-ECM interact actively with each other. The 3D microfluidic cell culture platform is considered to precisely control these external cues *in vitro*, thereby producing more reliable and predictive preclinical data than either animal models or conventional 2D cell-based models[5,20]. This is consistent with the trend toward more physiologically-relevant models, such as 3D organs or organoid-on-a-chips, for use in the early phase of drug discovery and development.

Over the past few decades, advances in microfluidic technologies have accelerated the development rate of the 3D cell culture or tissue model by virtue of the following significant features[19,24,25]. First, the microscale dimensions of microfluidic platforms are suitable for creating the biological microenvironment of *in vivo* tissues that have high complexity and spatial heterogeneity. Also, the physical structure of microfluidic channels can provide a well-controlled hydrodynamic environment, such as a chemical gradient or fluidic flow[26,27]. Second, the small scale of the systems requires only a small amount of cells and reagents in the experiments, which lowers the cost as the research progress from bio-analysis to drug development. Third, microfluidic technology can integrate the multiple and subsequent steps of bioanalysis, from culture and liquid handling to detection and analysis[28,29]. In addition, this technology is amenable to high-resolution, real-time monitoring, as well as the analysis of biochemical, genetic, and metabolic processes under conditions that closely resemble *in vivo* conditions. With these advantages, various approaches using microfluidic technology have been suggested in association with the study of stem cells, such as the cell culture, identification, and screening of cells as well as modeling diseases. We discuss the 3D microfluidic technologies in more detail because they provide potential solutions for problems in stem cell engineering.

3D MICROFLUIDICS IN STEM CELL ENGINEERING

Microfluidic chips provide a new platform with unique advantages to mimic complex physiological microenvironments *in vivo*. Since some groups started to use microfluidic technology for patterning or capturing stem cells in the early 2000s[30-32], the use of this technology in stem cell research has increased significantly. The emerging and rapid development of microfluidic technologies has presented an ideal solution in stem cell engineering, as summarized in Table 1. Many studies have been reported that focused on the application of microfluidic devices for stem cell research, such as culture, differentiation, patterning, tissue engineering, recreating organs, drug discovery, and therapeutics. In stem cell culture, it is important to control the biochemical microenvironment of cells to regulate the basic cell functions and biological processes, such as differentiation, development, and immune response. The temporal and spatial control over defined gradients of soluble factors or immobilized factors[33] provided by microchannel-based microfluidic devices can be an important advantage in stem cell research. For patterning cells or ECMs in desired locations, the patterned channel or the polydimethylsiloxane (PDMS) microwell generated using the soft lithography technique are simple and traditional method, while stably interfacing with other supporting cells[34,35]. The benefits of combining biomaterial engineering and microfluidics for stem cell applications are clear. Microfluidic technology could be used to mimic the spatial heterogeneity of stem cell microenvironment[36]. In particular, the chemical gradient in a microfluidic channel is one of the unique features that allows for this heterogeneous microenvironment. Some groups have used microfluidic approaches in which cells within hydrogels were exposed to desirable soluble gradients in 3D microenvironments[26,37]. Also, chemical gradient generators that use multiple microfluidic channels with flow control have been suggested to investigate the neural stem cell differentiation by the chemokine

(CXCL12) gradient generated within a single device[38]. To study cell-cell or cell-ECM interactions, the spatially-isolated compartments in a microfluidic device are also useful in investigating the differentiation or migration behavior of stem cells[39], and they help visualize their biological processes within a microscale device.

Despite the high potential impact of stem cell technologies, there are some technical challenges associated with culturing and differentiating stem cells for use in drug discovery and development. With a conventional well plate or dish, it is difficult to mimic the physiological complexity of the stem cell niche because it is a microenvironment that provides a variety of stimuli. Flow is one of the most important stimuli since some organs are affected by the shear flow induced by the blood stream. Microfluidic devices are the only platform capable of supplying flow, thereby inducing the important flow shear stress. This provides a way of observing stem cells by the effect of physical stimuli[40].

A 3D co-culture for niche construction can be achieved with droplet-based technology. By varying the ratio of the flow rates of the two cell streams, the ratio of the concentrations of the two types of cells can be altered within the microgel[41,42]. This technique enables the cells to be compartmentalized into a mono-dispersed and physicochemically-defined 3D matrix. Another advantage of this technique is its generation of high-throughput and microscale cell-matrix environments. For example, Sakai *et al*[41] reported the enclosed rat-adipose-derived stem cell aggregates in gelatin microbeads using a microfluidic droplet technique in which the stem cells were re-coated with additional supporting cells to construct a heterogeneous tissue structure.

Microfluidic devices combined with electrics and physics have been used to separate single cells[43-45]. Optical tweezers, electrical impedance, and dielectrophoresis techniques combined with microfluidic technologies can be used to sort or separate cells. For instance, Song *et al*[45] have developed a method to identify the differentiated state of human embryonic stem cells (ESCs) using electrical impedance in a microfluidic channel. Numerous other approaches have been tried by combining microfluidic technology with different analysis methods and by integrating various structures and functions. Recent advances in microfluidic technology using hydrodynamic trapping have resulted in an array culture method that enables precise and standardized tools that are controllable, constituent and high-throughput. Throughout the drug discovery and development process, a human stem cell-based cell culture system can be important in screening, validating candidate compounds and preclinical studies, such as the toxicity test, efficacy test, and the mechanism studies (*e.g.*, integration and automation[46-50], mechanical and electrical actuator[51,52]). Next, we discuss the microfluidic technologies in more detail for the high-throughput analysis of stem cells.

HIGH-THROUGHPUT ANALYSIS TECHNIQUES FOR STEM CELL ENGINEERING

The development of drugs requires a series of complex procedures that involves preclinical and clinical studies with well-established regulatory compliance. Developing a new drug, *i.e.*, from the discovery stage to approval by the United States Food and Drug Administration, generally takes more than 10 years and costs more than two billion dollars, and only about 10% of the compounds progress successfully through clinical development[6,20]. Current standard drug discovery traditionally starts with the 2D cell culture-based screening of compounds, followed by animal model testing and clinical trials. While 2D cell-based assays are used extensively because they have certain advantages, such as lower cost and higher throughput than animal tests, they also have limitations. These limitations include the lack of a cell-cell or a cell- ECM, which results in failure to reconstitute the *in vivo* cellular microenvironments, which means they cannot maintain the differentiated functions of the cells. Animal tests also cause errant pharmacokinetic predictions due to the differences between animal and human species that make it impossible to directly translate the findings in animal models to human biology. Therefore, there is a considerable need for new approach with a more accurate and cost-effective system that is representative of humans to efficiently screen and validate the potential drug candidates in the early stages of drug development[53].

Miniaturized, high-throughput techniques using microfluidics are required to identify efficient and cost-effective compounds using stem cell-based models and to gain insight into the possible underlying mechanism[23,54,55]. Microfluidic devices with micro-sized scale, automatic operation, and large-scale integration possibly can offer many unique benefits, including high-throughputs, low cost, and high efficiency in drug development. Also, due to the nature of microfluidic devices, quantitative

Table 1 Features of microfluidic techniques for stem cell engineering

Features of microfluidic technology	Solutions	Stem cell applications	Ref.
Cell or ECM patterning	Microchannel	Co-culture of hMSCs and hNSCs	Yang *et al*[34], 2015
	Microwell-patterned substrate	Co-culture of hESCs and fibroblasts	Khademhosseini *et al*[35], 2006
Chemical gradient	Multichannel array	Regulation of hematopoietic stem cell fates	Mahadik *et al*[26], 2014
	Overlapping gradients	Neuronal commitment of mouse ESCs	Cosson *et al*[37], 2013
	Sink and source channel with continuous flow	Chemotaxis of NSCs	Xu *et al*[38], 2013
Cell-cell or cell-ECM interaction	Microchannel-groove	Monitoring of differentiation and migration of NSCs derived from hESCs	Lee *et al*[39], 2013
Shear stress	Flow	Behavior observation of MSCs	Zheng *et al*[40], 2012
Droplet	Encapsulation and emulsion	Construction of ADSC microenvironment	Sakai *et al*[41], 2011
		Construction of pre-hatching embryo	Agarwal *et al*[42], 2013
Sorting and separation	Optical tweezer	Sorting of hESCs	Wang *et al*[43], 2013
	Electrical impedance flow	Identifying the differentiation of state of single cell	Song *et al*[44], 2013
	Multiple dielectrophoresis	Sorting of hMSCs	Song *et al*[45], 2015
Hydrodynamic trapping	Chamber array	EB-trap array	Suri, *et al*[46], 2013
Integration and automation	Multi-arrayed chips and integrated systems (*e.g.*, liquid handler, cell chamber, imaging system, software)	Automatic culture of stem cell-derived dopaminergic neurons	Kane *et al*[47], 2019
		Investigation of dynamic changes of hematopoietic stem cell condition	Dettinger *et al*[48], 2018
		Clonal analysis of hESCs differentiation pattern	Sikorski *et al*[49], 2015
Mechanical and electrical actuators	Mechanical stretch	Stretch-activated stem cell differentiation	He *et al*[51], 2018
	Magnetic resonance	Quantification of metabolic flux in leukemia stem cells	Jeong *et al*[52], 2017

ECMs: Extracellular matrixes; hMSC: Human mesenchymal stem cell; hESC: Human embryonic stem cell; NSC: Neural stem cell; ADSC: Adipose-derived stem cell; EB: Embryoid body.

analysis can be a useful tool in combinatorial mixing and processing samples[28,56,57] In drug discovery, HTS is a major instrumental technique. HTS commonly uses well plates ranging from 96 to 1536 plates, and these plates enable parallel and simultaneous testing of multiple factors. This allows rapid analysis of thousands of chemicals and biochemical using genetic or pharmacological tests in parallel, and this allows us to identify specific compounds for specific biological processes. Among these systems, the development of fast and automated microscopes, such as the high-content screening (HCS) microscope, has been accelerated by hardware advances and innovations in the software for analyzing images[13]. This system uses an automated liquid handler to simultaneously process hundreds of biological samples, and it provides the unbiased, multiple-parametric data with the high-spatiotemporal resolution from the acquired images, and it does so at the levels of individual proteins, organelles, whole cells, or even entire organisms. Therefore, this approach has been used to understand the complexity and dynamics of the cell biological processes that occur in cells and to identify a plethora of quantitative phenotypes of varying complexity in numerous different models.

With such advances in the scientific equipment, different approaches have been suggested for stem cell-based screening platforms using microfluidic devices. Table 2 provides a summary of some examples of microfluidic systems that have high-throughput capability for stem cell research. Miniaturization of the microfluidic platform increases the throughput of assays used to analyze stem cells because the small scale of the samples reduces both the consumption of reagents and the number of cells required[58-60]. Lee *et al*[61] and Du *et al*[57] suggested the microarray technique (1080 chips) and the microfluidic droplet array technique (342 droplets), respectively, for generating miniaturized cell array systems using cancer cell lines for the high-throughput testing of drugs. These techniques also can be applied to stem cell

research since they provide rapid and cost-effective testing for a wide range of applications that involve in high-throughput toxicity tests[60,62]. With the advent of robotic spotting technology and microfabrication, it is possible to generate the pattern of cells that are encapsulated in a 3D ECM matrix and that support cell growth at the microscale[59,62]. One of the powerful techniques of microfluidics for high-throughput 3D cell generation is the flow focusing technique, which is used for the encapsulation of cells in the ECM or hydrogel beads[42,63,64]. To understand the fate of stem cells, it is important to regulate the stem cell niche. Gobaa *et al*[65] reported microengineered niche spotting that was comprised of a hydrogel array for controlling the stiffness of the gel. As a similar example, Beachley *et al*[66] reported a 3D microtissue array when they used the spotting technique to investigate the tissue-specific response based on the composition of the ECM.

Also, soft lithography can be used to fabricate an array of wells with physically-defined dimensions, allowing for the cellular aggregates in the wells. The defined the sizes of wells can control the size of the cell aggregates and offer an attractive solution for controlling the fate of stem cells. Vrij *et al*[67] used optically-clear, cyclic olefin polymer (COP) films based on a thermoforming technique to develop a round-bottom, 96 microwell array for the generation of uniform-sized embryoid bodies (EBs). As a combined technique, arrayed microwell fabrication using PDMS soft lithography technique and droplet generation of cell suspension using surface tension due to hydrophobic and hydrophilic difference enable the formation of induced pluripotent stem cell (iPSC) arrays in a 512 well[68]. As another example, Occhetta *et al*[69] suggested that a high-throughput serial dilution generator could be used for making different concentrations and combinations of cytokine to investigate the effect of cytokines on the expansion and differentiation of embryonic stem cells (ESCs).

A major focus of the miniaturized HTS of stem cells is on screening for drugs, compounds and small molecules that could affect the properties of stem cells, such as differentiation, self-renewal, and expansion. 3D HTS platform also can be used to recreate the stem cell niche for mimicking the *in vivo* environment. Co-culturing different, interdependent types of cells is an important part of stem cell niche[70]. In general, HTS requires the use of robotics due to the multiple pipetting steps, and it consumes large quantities of reagents and valuable cells, resulting in the experiments having high costs. Despite the disadvantages, including the labor and time required, HTS technology using well plates is used extensively for developing various protocols for cell cultures and 2D and 3D screening of cells because the microplate is still a well-established platform for HTS applications so many research groups view it as a user-friendly approach. For this reason, Yu *et al*[71] developed the well plate-based gel unit array for HTS analysis. This platform has a unique feature in that it has hydrogel-incorporating compartments integrated in a well to culture 3D tissue with uniform thickness while co-culturing with other neighboring cells in a single well. This can be used as HCS integrated with a co-culture model.

3D TISSUE MODEL FOR STEM CELL ENGINEERING

Stem cells have their unique ability of self-renewal and the potential to differentiate into many specific types of cells. Immortalized cell lines are capable of extended proliferation but exhibit fewer organ-specific activities than primary cells or stem cells. Moreover, primary cells are functional, but have limited cell number and a finite lifespan. Therefore, stem cells that was able to differentiate into specific organs are considered to be more functional, and an ideal source to mimic the architecture and specific activity of human organs, and are more likely to be accurate with respect to human bodies. As a more reliable and sustainable human source that represents phenotypical characteristics of the inherited disease or genetic disorders, patient-specific cells are needed. Recently, iPSCs and their organoid techniques have undergone a rapid increase in popularity. These techniques allow reprogramming of fibroblasts into stem cells that can be differentiated into various tissues, such as neurons, cardiomyocytes (CM), and several types of blood cells. The iPSC technology provides a new and powerful tool for drug-screening for personalized medicines, and it allows the use of cells with the same genetic background as the patients. Furthermore, these sources of cells allow the recapitulation of various inherited diseases in vitro, and allow researchers to study the genotypic differences. For these reasons, iPSCs were have been used extensively in recent 3D *in vitro* organ models[22].

There are the two distinct strategies in generating *in vitro* 3D tissue and organ models, *i.e.*, the bottom-up and the top-town approaches[2]. A key example of the use of the top-down approaches is an organ-on-a-chip model, the aim of which is to engineer individual components of tissue environments, such as cells and ECMs in a

Table 2 High-throughput screening analysis for stem cell engineering

Techniques for high-throughput screening	Advantages	Applications	Cell types	Ref.
Microarray technique	Cell encapsulation in hydrogel-matrix spots; minimal consumption of cells and reagents	Toxicity and phenotypic screening of NPCs	NPCs	Nierode *et al*[60], 2016
		Studying of the expansion of mouse ESC	ESCs	Fernandes *et al*[62], 2009
Microencapsulation using microfluidic flow focusing	Multiple generation of 3D cells	Study of embryogenesis	iPSCs	Agarwal *et al*[42], 2013
	Encapsulation of cells and ECMs; controlled physicochemical properties of gel beads	Study of ESC expansion	mESCs	Allazetta *et al*[63], 2013
		Co-culture of pancreatic islets and hMSCs	hMSCs	Headen *et al*[64], 2014
Silicon stamp for spotting protein	Control of gel stiffness for stem cell fates	Study of stem cell niche	hMSCs	Gobaa *et al*[65], 2011
ECM array	Mimicking of microenvironments	Study of stem cell niche	hASCs	Beachley *et al*[66], 2015
Microwell array using thermoformed cyclic olefin polymer	Round-bottom array, uniform size of well array	EB generation	mESCs	Vrij *et al*[67], 2016
Micro droplet array; hydrophobic-hydrophilic surface	Robotics-free sample handling; high throughput; low reagent consumption; high-content readouts	Screening of iPSC pluripotency and proliferation	iPSCs	Zhang *et al*[68], 2016
Serial dilution generator	Generation of different concentration, combination and temporal sequence of drugs	Effect of cytokine (Tgfβ3) on hBM-MSC	hBM-MSC	Occhetta *et al*[69], 2015
Microraft array	Mimicking of microenvironments and enhancement of contact	Study of stem cell fate by mimicking niche	Intestinal stem cells	Gracz *et al*[70], 2015
Micropattern-well hybrid	Compatibility of HCS	Screening of stem cell differentiation and drug screening	NPCs	Yu *et al*[71], 2018

ECMs: Extracellular matrixes; iPSC: Induced pluripotent stem cell; NPC: Neural progenitor cell; mESC: Mouse embryonic stem cell; hASC: Human adipose-derived stem cell; HCS: High-content screening.

microfluidic device, and this work is conducted mostly by bioengineers. Bottom-up approaches rely on biological self-organization, which refers to intrinsic abilities of biological systems, and they are led largely by stem cell biologists. These two approaches both have the same goal, *i.e.*, achieving the generation of high-fidelity 3D tissue. However, both approaches have their own limitations. For instance, organoid systems have low controllability for recreating the biochemical and biophysical microenvironment of 3D organoids, while organ-on-a-chip systems have limitations when reconstituting the biological complexity of tissue development. Thus, by combining the strengths of both two approaches, the organoid-on-a-chip platform has emerged as a synergistic approach to recapitulate both the physiological and biochemical features of *in vivo* tissue[10,14]. In this section, we introduce examples of stem cell-based organ-on-a-chip and organoid-on-a-chip system using microfluidic technologies for high-throughput analysis.

ORGAN-ON-A-CHIP AND ORGANOID-ON-A-CHIP FOR HIGH-THROUGHPUT ANALYSIS

With the development of the generation of iPSCs, tissue models and disease models based on organ-on-a-chip technology have been proposed, and they are expected to serve as a platform for cell-based, high-throughput assays during the drug discovery and development. The organ-on-a-chip, which utilizes the microfluidic approach to mimic the architecture and function of 3D tissue, consists of microengineered biomimetic systems that represent key functional units of living human organs. Also, recent advances in microfabrication, cell engineering, and imaging technologies have led organ-on-a-chip to become an innovative technology that is capable of reproducing physiological cell behaviors *in vitro*. These systems include important

design considerations for developing systems, *i.e.*, (1) Organizing the spatial distribution of multiple types of tissues; (2) functional tissue-tissue interfaces; and (3) organ-specific mechanical and biochemical microenvironments.

Stem cell-derived organoid systems that are 3D self-organized tissue models provide new biological models for the development of new drugs. Organoids have been generated from both pluripotent stem cells and tissue-resident adult stem cells by mimicking the biochemical and physical cues of tissue development and homeostasis[72,73]. Because of these unique features, conventional 3D organoid systems may be more advantageous in some aspects than organ-on-a-chip systems in drug discovery. One of the important applications of organoid cultures is to model pathologies of diseases. Organoid-on-a-chip engineering has been emerged recently based on the integration of the two distinct approaches of organoid and chip technology.

Stem cells, including iPSCs, have the potential to serve as a source of cells that can be engineered to suit specific needs in the development of organ-on-chips[5,6]. In recent years, the organ- and organoid-on-a-chip approaches using stem cells have been used extensively to establish the new microengineered models that recapitulate the structure and functional complexity of human organs, such as the liver[74-77], heart[78-86], brain[71,87-94], intestine[95-97], kidney[98-100], and bone[101-103]. Recently, organ-on-a chip technology has been able to integrate multiple organ or tissue models to simulate the human body, and multi-organ systems generated using stem cells have been developed for a human body-on-a chip system[16,75,104,105] It is possible for such a system to provide a predictive model for pharmacokinetics of drugs by mimicking the activities of the human body such as absorbing, distributing, metabolizing, and eliminating drugs.

We introduce examples of organ- and organoid-on-a-chip platform using stem cells for high-throughput assay, as summarized in Table 3. In general, the primary focus of organ-on-a-chip has been on the microengineered liver due to the importance of its central role relative to hepatic drug toxicity and metabolism[74-76]. Ware *et al*[74] demonstrated the possibility of a high-throughput hepatotoxicity test on iPSC-derived hepatocytes co-cultured with fibroblasts, which were micropatternd islands using the soft-lithography technique. In another approach, Schepers *et al*[76] developed a liver-on-a-chip using human iPSCs from a patient. This cell was cultured as 3D organoids using a perfusable system, and the organoids that were constructed were integrated in a chip with multiple patterned C-traps, as shown in Figure 1A. The liver organoids were long-term cultured for 28 d during perfusion. Recently, researchers have begun to explore the potential of heart-on-a-chip as a HTS tool for the monitoring of contractile functions and cardiomyopathy using iPSC-derived CMs[83,84,86]. For instance, Mills *et al*[86] developed a 96 well-type screening platform for screening functions in hiPSC-derived cardiac organoids to reveal the cardiac metabolic mechanism, as shown in Figure 1B.

Similarly, there have been recent developments in brain-on-a-chip for HTS. The attention for brain models fits in a wider trend towards attention for neural progenitor-cell-derived brain models for diseases, such as Alzheimer's disease[71,91]. This follows the more generic increase in the popularity of iPSC techniques and progress in controlling the stem cell niche of differentiated tissues. Wang *et al*[93], developed brain-organoids using iPSCs to model neurodevelopment disorders under prenatal nicotine exposure and showed the potential of drug testing. In addition, the 96-well plate-based, HTS-compatible 3D cell culture platform for the brain model was developed for preclinical drug screening applications[71,92]. Especially, Yu *et al*[71] developed a micropattern array platform combined with conventional well-plate for HTS drug screening to show the proof-of-concept for the Alzheimer's disease model using neural progenitor cells, as shown in Figure 1C. Wevers *et al*[94] showed 3D ECM-embedded neuronal-glial networks in a microfluidic platform using iPSC-derived neural stem cells. The iPSC-derived mature neurons and astrocytes were cultured in the microfluidic channel-based OrganoPlate, which is the integrated microtiter plate that is comprised of 96 tissue chips developed by MIMETAS, Inc., as shown in Figure 1D. An HTS-compatible platform also was developed in kidney-on-a-chip by Czerniecki *et al*[100]. The iPSCs were cultured and differentiated on this platform with fully-automated and HTS-compatible formats for multi-dimensional phenotypic screening.

CHALLENGES AND FUTURE PROSPECTS

In the field of drug screening, the need for a 3D stem cell platform will become more pressing because it provides a more efficient approach in the early, preclinical stage of

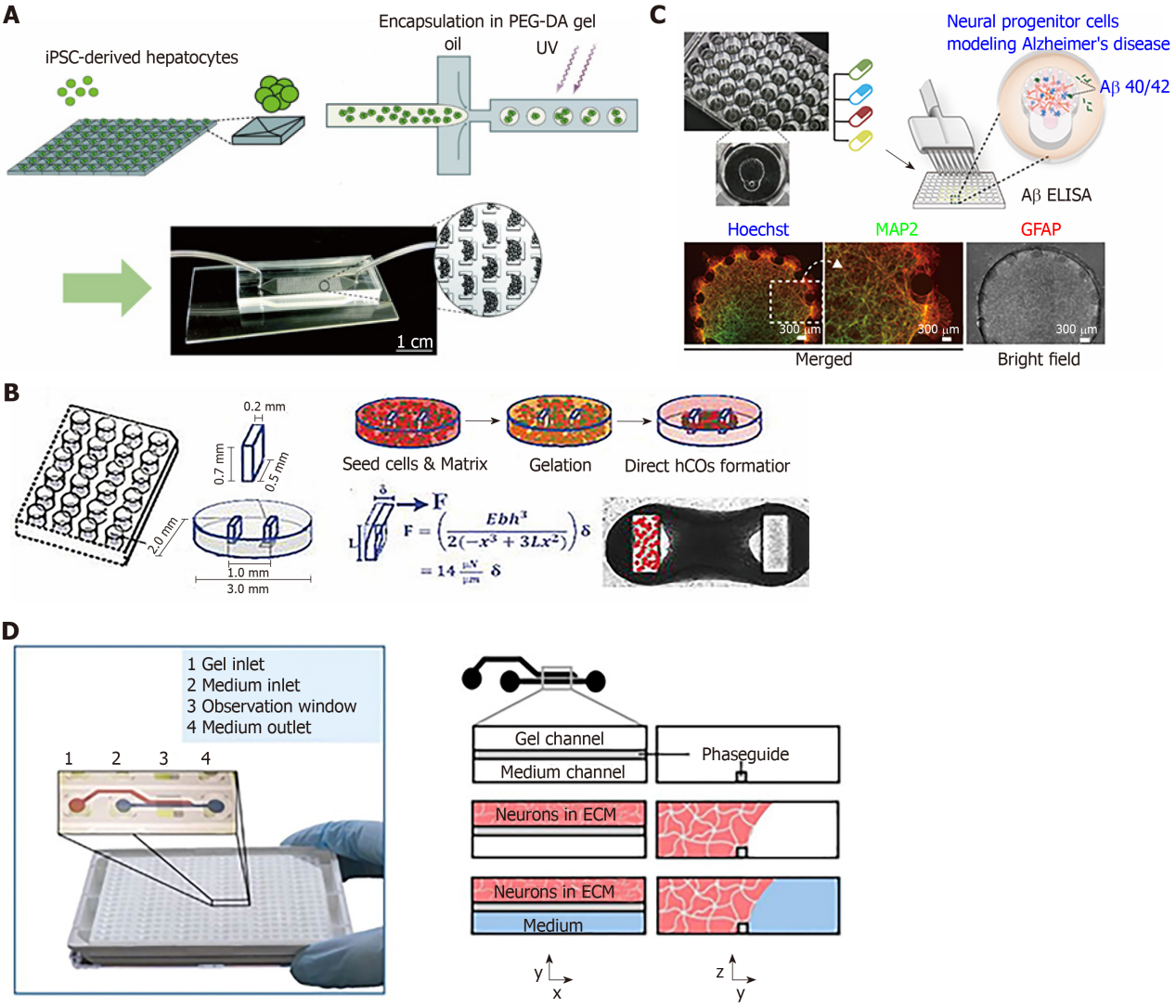

Figure 1 Representative examples of high-throughput screening microfluidic systems using stem cells based on organ-on-a-chip or organoid-on-a-chip. A: An engineered perfusable liver platform using induced pluripotent stem cell (iPSC). The iPSC-derived hepatocytes were aggregated as three-dimensional (3D) organoids and encapsulated in Poly (ethylene glycol) diacrylate hydrogel. These cells were loaded in a C-trap chip subjected to perfusion for a long-term culture (Reproduced from Ref[76] with permission from the Royal Society of Chemistry); B: A miniaturized 96 well-type human iPSC-derived cardiac organoid (hCOs) screening platform, which is called heart dynamometer (Heart-Dyno), facilitates the automated formation of hCOs (Reproduced from Ref[86] with permission from the National Academy of Science); C: Well plate-micropattern hybrid platform for NPC differentiation for modeling Alzheimer's disease (Reproduced from Ref[71] with permission form the Royal Society of Chemistry). This hybrid-platform is compatible with high-throughput screeninganalysis; D: Organo-plate® comprising 96 microfluidic tissue chips and experimental outline for culturing 3D neuronal-glial networks (reproduced from Ref[94] with permission from the Nature Research).

drug development. Although 3D microfluidic technology provides significant potential for creating a highly complex, well-controlled 3D dynamic environment as an *in vivo* system, there are certain to be technical challenges in both the engineering and biological technologies of this platform. In general, microfabricated devices contain various complex designs within small areas, and this limits biochemical experiments and requires advanced skill and optimization. Under such physical conditions, certain types of stem cells can be very sensitive to the excessively high shear stress induced by flow, which might cause phenotypic changes or adversely affect cell viability in microfluidic devices during long-term cultures[106]. In addition, high adsorption of proteins on the PDMS or the plastic walls of microfluidic devices also can hinder the accurate evaluation of the effects of drugs. Currently, several of these problems are being addressed by simpler designs, and stem-cell-specific changes in the design of the devices. Recent approaches that have relatively simpler hybrid systems that combine traditional cell culture plates with microfluidic compartments by decoupling the handling of cells from handling of microfluidic liquids could be alternative approaches[37]. Also, the various microfluidic designs, such as low perfusion, deeper chambers, and large input/output reservoirs to avoid handling the tubes, could be solutions.

In the aspects of high-throughput analysis, the nature of microfluidic systems,

Table 3 High-throughput screening-based three-dimensional organ- or organoid-on-a-chip

Organ type	Platform type	Cell type	Applications	Ref.
Liver	Organ-on-a-chip	Human iPSC -hepatocytes	Screening of hepatotoxic drugs	Ware *et al*[74], 2015
	Organ-on-a-chip	Hepatocyte-ESC line	Co-culture of multi-organ	Materne *et al*[75], 2015
	Organoid-on-a-chip	iPSCs	Tests of liver function	Schepers *et al*[76], 2016
Heart	Organ-on-a-chip	Human iPSC - cardiomyocytes	Screening of molecular inducer related to cardiac myocyte proliferation	Titmarsh *et al*[83], 2016
		Human iPSC - cardiomyocytes	Modeling of mitochondrial cardiomyopathy of Barth syndrome	Wang *et al*[84], 2014
	Organoid-on-a-chip	hPSCs	Study of cardiac maturation	Mills *et al*[86], 2017
Brain	Organ-on-a-hip	NPCs	Toxicity test with *in vitro* brain model of Alzheimer's disease	Park *et al*[91], 2015
		NPCs	Calcium assay	Lai *et al*[92], 2012
		NPCs	*In vitro* test for Alzheimer's disease	Yu *et al*[71], 2018
	Organoid-on-a-chip	Human iPSC	Model of neurodevelopment disorder by prenatal nicotine exposure	Wang *et al*[93], 2018
	Organ-on-a-chip	Human iPSC	High-throughput compound evaluation on three-dimensional networks of neurons and glia	Wevers *et al*[94], 2016
Intestine	Organoid-on-a-chip	Human iPSC	Study of response to exogenous stimuli	Workman *et al*[97], 2018
Kidney	Organoid-on-a-chip	Human iPSC	High-throughput screening format organoids for multidimensional phenotypic screening	Czerniecki *et al*[100], 2018

iPSC: Induced pluripotent stem cell; NPC: Neural progenitor cell; ESC: Embryonic stem cell.

which require complicated handling and multiple processes for a series of biological processes, present barriers to high-throughput analysis[19,28,50,53]. This is especially important in the case of primary patient-derived stem cells with time constraints that could be cultured outside of the organism due to rapid changes in their microenvironments during *in vitro* culture. For this reason, miniaturized screening compartments, systemized cell manipulation, and robotic liquid handling must be developed.

In addition, many proposed systems, as with many other HTS platforms, are focusing largely relying on biomolecular engineering techniques coupled with microscopy-based imaging. However, practical *in vitro* systems require a system that both observe and analyze a variety of biochemical and physiological responses[54].

Despite of these challenging issues, the high demand for microfluidic devices for HTS of stem cells is uncontroversial. Microfluidic technology is still evolving to overcome these current issues, and the techniques are becoming more sophisticated and acceptable for miniaturization, automation, and versatile testing of all critical parameters for stem cell research. The combination of microfluidic technologies with stem cell analysis may fill the gaps between the present knowledge about stem cells and an in-depth understanding of the underlying mechanisms for their broad applications. By using these techniques in the future, *in-vivo*-like culture of stem cells and their drug discovery applications can be improved, and the prediction of drug responses will be more reliable.

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REVIEW

Neural stem cell transplantation therapy for brain ischemic stroke: Review and perspectives

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Abstract

Brain ischemic stroke is one of the most common causes of death and disability, currently has no efficient therapeutic strategy in clinic. Due to irreversible functional neurons loss and neural tissue injury, stem cell transplantation may be the most promising treatment approach. Neural stem cells (NSCs) as the special type of stem cells only exist in the nervous system, can differentiate into neurons, astrocytes, and oligodendrocytes, and have the abilities to compensate insufficient endogenous nerve cells and improve the inflammatory microenvironment of cell survival. In this review, we focused on the important role of NSCs therapy for brain ischemic stroke, mainly introduced the methods of optimizing the therapeutic efficacy of NSC transplantation, such as transfection and overexpression of specific genes, pretreatment of NSCs with inflammatory factors, and co-transplantation with cytokines. Next, we discussed the potential problems of NSC transplantation which seriously limited their rapid clinical transformation and application. Finally, we expected a new research topic in the field of stem cell research. Based on the bystander effect, exosomes derived from NSCs can overcome many of the risks and difficulties associated with cell therapy. Thus, as natural seed resource of nervous system, NSCs-based cell-free treatment is a newly therapy strategy, will play more important role in treating ischemic stroke in the future.

Key words: Ischemic stroke; Neural stem cells; Transplantation; Cytokines; Exosomes

Core tip: In this review we compiled the latest available research regarding the use of neural stem cell therapy for the treatment of brain ischemic stroke. We discussed the benefits and limitations of this type of therapy focusing on the current efforts to improve

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its safety and efficacy. Further, we described a novel and clinically relevant strategy for the treatment of ischemic stroke based on cell free treatment–exosomes.

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BRAIN ISCHEMIC STROKE

Globally, stroke is one of the top three common causes of death and disability. It is divided into the following two types: Ischemic and hemorrhagic stroke, of which ischemic stroke accounts for more than three-quarters of cases (about 80%-85%)[1]. Following stroke onset, patients can suffer from various neurological dysfunctions that renders them unable to take care of themselves. This seriously affects their quality of life, impacts on their patients and their families physically and mentally, as well socially and economically. Recently, numerous preclinical and clinical studies[2,3] have discovered many drugs or molecules that may potentially exert certain beneficial effects in the treatment of ischemic stroke; however, these drugs exhibit limited or no therapeutic efficacy under clinical applications; thus, a novel drug for this purpose still needs to be explored.

Ischemic stroke can induce some harmful pathophysiological changes around the ischemic area of the brain[4-6]. This event is mainly characterized as acute ischemia and hypoxia of nerve tissue after blood interruption to local brain tissue in a short period of time. This is followed by a series of cascade reactions, including the production of local oxygen free radicals and reactive oxygen species, increase in local tissue permeability, generation of cerebral edema, local immune inflammatory cell infiltration, development of neuroinflammation, and blood-brain barrier destruction. These lesions significantly expand in a short period of time, leading to local nerve cell damage and necrosis, reduction of functional neurons, and axon network disruption. Subsequently, due to the extremely poor microenvironment around the peri-ischemic cell regions, secondary brain damage occurs, resulting in an irreversible neural tissue injury. The limits self-repair of nerve tissue. Even after recovery of blood flow to the ischemic area, enough nerve cells and an appropriate immune microenvironment to supplement and repair the functional nerve tissue that died are still not available[4-6]. This eventually leads to permanent local nerve tissue loss and nerve function defects of the brain (Figure 1).

However, the treatment of brain ischemic stroke is individualized and involves heterogeneous approaches, most of which are closely related to the location of ischemia, the age of patients, and the ability of local nerve self-repair. The main purpose of clinical treatment of stroke is to restore local cerebral blood perfusion and reduce the occurrence and degree of disability or dysfunction after stroke onset as early as possible[2,7]. Due to the weaker regeneration ability of endogenous nerve cells and the harmful effects of an ischemic microenvironment, there are currently no effective methods or strategies available for treatment. Recombinant tissue plasminogen activator (r-tPA) is the only effective class 1A recommended drug for the treatment of acute ischemic stroke approved by the American Food and Drug Administration, but it has a very narrow treatment window (generally within 4.5 h, and no more than 6 h) and it has various treatment-related contraindications or complications (such as increased risk of cerebral hemorrhage)[8-10]. Therefore, its application has been markedly limited; only a small percentage of patients (no more than 5% of patients) can receive this timely and effective treatment. In addition, intravascular interventional therapy (such as endovascular thrombectomy) can be used as an adjuvant or replacement therapy for early reocclusion of large vessels in patients with contraindications or complications of intravenous thrombolysis[11-13]. This adjuvant therapy can extend the time window of potential treatment to 12 h, and it can also improve the functional independence and vascular remodeling rate of the patient compared to tPA treatment alone. However, the treatment time still has an important effect on the efficacy of this intravascular treatment, because just a 30 min delay beyond the timeframe can significantly reduce the functional independence of patients. Moreover, current endovascular interventions are still difficult to generalize, since only regional stroke centers with neurological intervention capabilities can

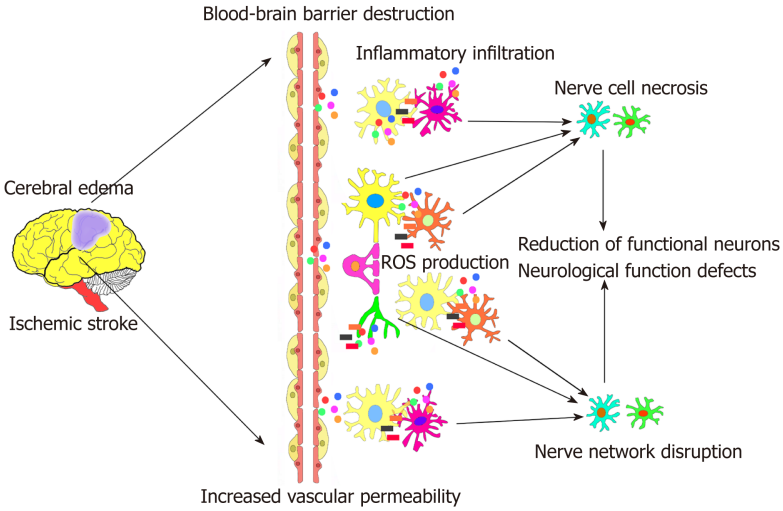

Figure 1 The pathological processes of brain ischemic stroke. After blood was interrupted in local brain tissue, there was a series of cascade reactions following by: BBB destruction, vascular permeability increased, cerebral edema generation, inflammatory factors infiltration, oxygen free radicals and ROS production, neuro-inflammation occurrence *etc*, induced local nerve cell necrosis and axon network disruption, resulting in reduction of functional neurons and an irreversible neurological function defects of the brain. BBB: Blood-brain barrier; ROS: Reactive oxygen species.

perform this operation, and only less than 10% of stroke patients are eligible to undergo this effective treatment[11,14].

Thus, treatment of subacute and chronic phases of stroke is important, and it is mainly based on the clinical rehabilitation of patients with symptoms (such as different rehabilitation methods according to different stages and dysfunctions)[2,7,15-17]. This includes subject stroke unit nursing, home or hospital rehabilitation training, task-oriented training, mandatory exercise therapy, different high-intensity training, repetitive task training, spatial sensation, and language ability training. Although the abovementioned rehabilitation methods can improve the symptoms of some patients to a certain extent after long-term adherence, the effect of rehabilitation is limited, and it is still not enough to improve the overall prognosis of stroke patients. Therefore, the therapeutic prognosis of patients with brain ischemic stroke is closely related to the permanent loss of local functional neurons and networks, the repair of local glial scars, and the lack of activated endogenous neural stem cells[7,18-20].

NEURAL STEM CELLS

In 1992, Reynolds *et al*[21] isolated a cell population with self-renewal abilities and a multi-directional differentiation potential from the striatum of adult mice; these were proposed as neural stem cells (NSCs). Subsequently, in 1997, Mckay *et al*[22] officially defined the concept of NSCs as a self-renewing cell population with pluripotent abilities that can differentiate into neurons, astrocytes, and oligodendrocytes. Recent studies[23,24] have confirmed that NSCs are present in the lateral ventricle (subventricular zone, SVZ) and hippocampal dentate gyrus (subgranular zone, SGZ) of adult animals. NSCs in the SVZ region mainly migrate along the rostral migratory stream to the olfactory bulb, whereas stem cells in the SGZ migrate to the granule cell layer, and finally differentiate into various neural cells and integrate into nerve networks[23-26]. In addition, with the exception of SVZ and SGZ, the human brain possibly consists of another stem cell pool in the deep ventral region of the prefrontal cortex, due to the highly developed prefrontal lobe in the human brain.

Furthermore, endogenous NSCs exist *in vivo*, whereas exogenous NSCs are cultured *in vitro*. Under normal conditions, endogenous NSCs in the body are in a static, undifferentiated dormant state (called quiescent NSCs, qNSCs), and maintain a dynamic balance in the stem cell pools[27-29]. Once they are exposed to external stimuli such as brain damage, qNSCs can be activated to proliferate, migrate, and differentiate, thereby participating in the repair process of damaged nerve tissue[29-31]. Usually, the number of endogenous cells activated is very limited, and a large proportion normally differentiates into glial cells. Glial scars are involved in tissue repair, so that the loss of functional neurons (neural network) is not enough to supplement[32-34]. Moreover, significant neurological dysfunction still persist for a long

time after brain injury, indicating that the neuroregenerative ability of endogenous cells is largely insufficient, and is not enough to replace damaged functional nerve tissue[7,35,36].

With the development of stem cell therapy research, adult stem cells have received more and more attention and provide new directions for future clinical treatment of refractory diseases including ischemic stroke[37-40]. NSCs have many advantages, such as self-renewal, low immunogenicity, and good histocompatibility, as well as multi-directional differentiation potential; they can differentiate into three types of nerve cells to maintain and repair damaged brain tissue. Thus, NSCs act as a natural active resource and are considered to be a good tool for treating nervous system diseases. Numerous preclinical studies[39,40] have found that transplantation of exogenous NSCs can significantly complement or replace damaged tissues and treat various neurological diseases. Studies[36,41-44] have also shown that after treatment of neurological diseases with exogenous NSC transplantation, the deficiency of endogenous NSCs was not only supplemented *in vivo*, but the immune micro-environment around the tissue injury area was also improved. Many preclinical studies[45-47] have demonstrated that exogenous NSCs have a certain therapeutic effect in neurological diseases, and certain studies have found that the therapeutic functions of exogenous NSCs can be enhanced by combining or overexpressing them with some cytokines [such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), or NGF *etc*]. However, the efficiency of exogenous stem cell transplantation therapy is still controversial, mainly due to the low grafting efficiency of exogenous stem cells in the brain (less than 5%). Furthermore, the inflammatory immune microenvironment is very severe. Nowadays, the mechanisms of treatment with stem cell transplantation are not entirely clear, and further research methods are needed.

TRANSPLANTED NEURAL STEM CELLS THERAPY FOR BRAIN ISCHEMIC STROKE

Due to the ethical, therapeutic efficacy, and safety issues, exogenous NSC transplantation therapies have a long way to go to reach the stage of clinical application. At present, only a few clinical studies have been conducted; however, a number of preclinical animal experiments have been conducted[38,40,48-50]. A large number of these studies[37-40] have evaluated the therapeutic efficacy and safety of transplanted exogenous NSCs in preclinical animals with brain ischemic stroke. Their results demonstrated that exogenous NSCs could significantly improve the prognosis of cerebral ischemic animals, and not only the functional outcome was improved, but also the histological infarct volume was significantly reduced, with no obvious security issues. The following two main mechanisms have been approved for the activity of exogenous NSCs in the treatment of brain ischemic stroke[36,41-44,51,52] according to the first mechanism, transplantation of exogenous stem cells compensate for the deficiency of endogenous stem cells and activate more endogenous cells to repair the nerve damage; the second mechanism reveals that transplantation of exogenous stem cells improves the inflammatory immune microenvironment around the ischemic regions, which then mediates neural network reconstruction based on the bystander effect (Figure 2).

Animal experiments

Currently, the results of NSC transplantation for brain ischemic stroke in animal models are satisfactory. Moreover, the efficacy and safety of stem cell transplantation has also been confirmed. Lees *et al*[53] and Vu *et al*[54] used meta-analyses to evaluate the therapeutic efficacy of stem cell transplantation (including NSCs) in 117 and 46 preclinical animal models with cerebral ischemic stroke, respectively. After treatment, the neurological function of cerebral ischemic animals is improved significantly, and the volume of cerebral infarction reduced. Furthermore, the degree of prognosis improvement was correlated with the source of stem cells, injection route, injection timing, and dose of injection[53,54]. Chen *et al*[40] collated and analyzed animal studies of NSC therapy for the treatment of brain ischemic stroke. A total of 37 studies and 54 independent intervention groups were analyzed and meta-analyzed. The results showed that transplantation of NSCs significantly improved neurological function and histological structure outcomes of cerebral ischemic animals. Of the studies analyzed, 36 reported neurological improvement, 22 reported improved histology, and 21 reported beneficial outcomes in both neurological function and histological structure. They also found that the degree of improvement in prognosis function of ischemic animals had a certain correlation with the injection time of NSCs, the source

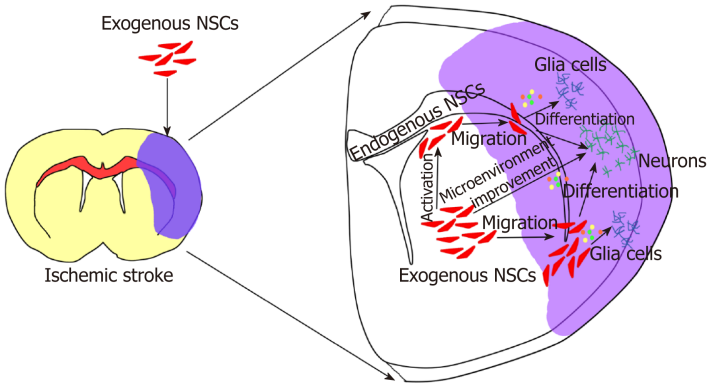

Figure 2 Transplanted exogenous neural stem cells therapy for brain ischemic stroke. After exogenous NSCs were transplanted into the ischemic brain, they can induce the endogenous NSCs activation of SVZ region. Then both exogenous and endogenous NSCs migrated to the cortical site of ischemic injury, differentiated to glia cells and neurons which contributed to glial scar formation and nerve tissue repair. Furthermore, it had two main mechanisms for reconstructing ischemic neural tissue *in vivo*: exogenous and activated endogenous stem cells directly compensated the lost nerve tissue by differentiating into nerve cells, transplanted NSCs improved the harmful inflammatory microenvironment around the ischemic regions *via* the bystander effect. NSCs: Neural stem cells; SVZ: Subventricular zone.

of stem cells, and whether immunosuppressive agents had been used[40]. No significant safety problems were found. Although some differences in research quality and different degrees of publication bias between the different animal experiments exist[55-59], the overall results suggest that NSCs can effectively improve neurological function of cerebral ischemic stroke animals. They can reduce the area of ischemic infarction, proliferate, migrate, and differentiate into neurons *in vivo*. In addition, some neurons may be integrated into the neural network of the brain.

Clinical trials

At present, there are very few clinical trials with NSCs registered in the Clinical Trial Database (ClinicalTrials.gov) for brain ischemic stroke. The first study is a Phase II clinical trial (NCT02117635) conducted at the Queen Elizabeth University Hospital of the University of Glasgow, UK. The current status of the trial shows that it has been completed, but no publications have been made for the Phase II clinical trial to date. They published the Phase I clinical trial (NCT01151124) of human NSC therapy for chronic cerebral ischemic stroke in the Lancet issue from August 2016[38]. This clinical study was an unblinded, single-center transplantation study that involved different doses of CTX0E03 cells (an immortalized NSC line, ReNeuron). Allogeneic transplantation of immortalized NSCs CTX0E03 was performed by stereotactic injection. A total of 11 patients were enrolled. Four groups were injected with different doses of exogenous NSCs. The patients were followed up for 2 years and evaluated by the NIHSS (National Institute of Health stroke scale) and Barthel Index methods. All of the evaluated methods and MRI images confirmed that the exogenous NSCs significantly improved the neurological function of patients after NSC transplantation, with no related adverse reactions[38]. This study was the first exogenous NSC transplantation performed in clinical patients with brain ischemic stroke[38]. None of the patients used immunosuppressants due to human leukocyte antigen (HLA) mismatch, and the results showed that some patients had significant neurological improvement. However, because the number of patients included in a Phase I trial are small, and no control or placebo groups are enrolled (which may relate to ethical issues), more detailed data in the Phase II clinical trial are expected. The second study is a Phase I clinical trial registered with Beijing Bayi Brain Hospital (NCT03296618). In this study, Exogenous NSCs were used from Neuralstem's NSI-566 cell line derived from primary human fetal spinal cord tissue. The results of this Phase I clinical trial have not yet been published.

OPTIMIZING THE THERAPEUTIC EFFICACY OF NEURAL STEM CELL TRANSPLANTATION

Although preclinical studies have confirmed the efficacy and safety of NSC transplantation for the treatment of ischemic stroke, there are still some controversies. Since the grafting efficiency or survival rate of stem cells is less than 5% *in vivo*, there

are still many problems that should to be addressed before this treatment can be used for clinical applications[60-63]. The most important issue is the grafting efficiency and differentiation ratio of NSCs *in vivo* after transplantation. In addition, both endogenous and exogenous NSCs differentiate into glial cells *in vivo* in a significantly higher ratio than that of neurons[64-66]. Thus, many studies have attempted to modify the gene expressions or protein levels of NSCs using different strategies such as virus transfection to express specific genes, pretreatment of cells with inflammatory immune factors, and combination with cytokines to increase the therapeutic effects of transplanted cells.

Gene overexpression

BDNF can promote the differentiation of transplanted NSCs into neurons and increase their survival[67,68]. Therefore, studies have attempted to overexpress the BDNF gene in NSCs for improving the therapeutic potential of stem cells *in vivo*[67,68]. After transplantation of human NSCs that overexpress the BDNF gene in the ischemic striatal region of MCAO rats, the contralaterally transplanted NSCs were found to migrate to the infarcted area *via* MRI images. Neurobehavioral functions of ischemic rats were also significantly improved, and the transplanted cells co-localized with Nestin, DCX, and MAP2 positive cells, indicating that the transplanted NSCs participated in nerve regeneration and functional recovery *in vivo*. Lastly, protein expression of BDNF was also high in the ischemic regions[46].

Neurotrophin-3 (NT-3) belongs to a family of neurotrophic factors and has been found to be involved in mediating stem cell survival and inducing neural differentiation[69,70]. The lentiviral vector (LV) encoding human NT-3 was constructed and transfected into NSCs, and then transplanted into the ipsilateral striatum region of MCAO rats. After 2 to 4 wk of transplantation, secretion of NT-3 protein was significantly higher than that in the control group. Concurrently, the neurobehavioral function in cerebral ischemia rats was significantly improved, when compared to the control group[71].

VEGF is an important angiogenic factor, and it is involved in mediating angiogenesis and nutrient supplementation[72,73]. After transplantation of NSCs transfected with the VEGF121 gene into the ischemic surroundings of stroke rats, studies found that the cells survived and migrated in the ischemic area for up to 12 wk after transplantation. At the same time, the neurological function of the cerebral ischemia rats was significantly improved, compared to the untransfected NSC group, indicating that VEGF121 transfection additionally increased the therapeutic efficacy of transplanted NSCs[74].

The above studies suggest that exogenous genes can be introduced into NSCs by LVs. The modified NSCs carry the therapeutic-related genes to the damaged areas and can express them effectively, and finally increasing the repairing effects of transplanted NSCs.

Preconditioning of NSCs with cytokines

Although overexpression of genes in NSCs leads to better transplantation outcomes by promoting trophic or survival signaling to cells, more beneficial, simpler, and safer methods need to be developed for future clinical applications. Consequently, studies have introduced immunological correlations where NSCs are pretreated with cytokines or inflammatory factors for treatment[75,76].

Interleukin-6 (IL-6) is a pro-inflammatory cytokine involved in the pathogenesis of various neurological diseases including stroke[77,78]. Serum IL-6 levels in patients with clinical ischemic stroke are associated with poor infarct volume and long-term prognosis[79]. When NSCs were pretreated with IL-6, results showed that stem cells were reprogrammed, and the signal transduction and transcription activator 3-mediated manganese superoxide dismutase (SOD2) was significantly up-regulated in the cells[60]. Expression of SOD2 promoted cell survival in the ischemic area; pretreated stem cells also induced the secretion of VEGF, promoted microvascularization, and significantly reduced the infarct size of cerebral ischemia, as well as improved neurological functions. These results demonstrated that pretreatment with IL-6 can properly reprogram NSCs to withstand the oxidative stress environment after an ischemia-reperfusion injury, induce angiogenesis, and ultimately improve the effectiveness of transplanted stem cells for the treatment of ischemic stroke[60].

In addition, expression of inflammatory cytokines such as IL-1, IL-15, and Interferon-gamma (IFN-γ) normally increase after central nervous system injury. When NSCs were pretreated with anti-inflammatory factors such as IL-4 or IL-10, the results were consistent with those using a pretreatment of IL-6. These cytokines mainly played a neuroprotective role and promoted the migration of cells to the injury site. The pretreatment method dominantly provided a more favorable microenvironment for the proliferation of NSCs after transplantation[80,81].

Additionally, BDNF-pretreated with NSCs produced similar results, where increased cell survival, migration, and improved neurological function were seen in cerebral ischemic animals after transplantation[82].

Thus, a better understanding of the relationship between neuro-inflammation and neurogenesis, and an understanding of the potential mechanisms of inflammatory stimulation in cerebral ischemia is essential. *In vitro* pretreated stem cells with cytokines or inflammatory factors may further induce the migration of NSCs to inflammatory regions, increase the neuroprotection of NSCs, and more effectively increase the therapeutic effects of stem cells.

Co-transplantation with factors

Cytokines can regulate the self-renewal, proliferation, and differentiation of stem cells, but to maximize the therapeutic potential of stem cells and ameliorate the damage, regulation of the microenvironment may be crucial. Currently, the main direction of NSC-based research is to explore new tools for nerve regeneration. Viral vectors and gene therapy may have certain deficiencies, such as potential tumor formation and lack of efficiency. Studies[81,83,84] have attempted to deliver therapeutic drugs through implanted pumps for sustained release, but deficiencies still persist with these strategies.

Neurotrophic factors can increase the survival of NSCs and promote their proliferation or differentiation. VEGF plays various roles in the CNS, including pro-angiogenesis, neurogenesis, and neurotrophic and neuroprotective effects[73,85,86]. Study have attempted to investigate the feasibility of co-administration of VEGF with human NSCs[87]. The results showed that VEGF and transplanted NSCs had a certain synergistic effect in cerebral ischemia. The combination-treatment group expressed a better behavioral recovery than single-treatment groups, and the degree of brain atrophy in the cerebral cortex and striatum was significantly decreased. However, the distribution of VEGF was not co-localized with NSCs, suggesting that VEGF promoted the therapeutic efficacy of NSC transplantation through pro-angiogenic effects[87]. IFN-γ is a mediator of the pro-inflammatory pathway and plays an important role in the ischemic brain. However, IFN-γ does not hamper the ability of NSCs *in vitro*. Study[88] have shown that co-delivery of IFN-γ (50 ng) enhanced the effects of transplanted NSCs in ischemic rats. The study found that in the combination-treatment group, neurogenesis was significantly increased when compared to other groups *in vivo*. Moreover, co-treatment with IFN-γ and NSCs provided additional beneficial neurological outcomes. Thus, low concentrations of IFN-γ can mediate NSC functions and facilitate their ability for neurological repair[88].

In addition, vascular progenitor cells (VPC) was co-transplanted with NSCs. It was found that co-transplantation of NSCs and VPC enhanced the differentiation ratio of neurons and microvessel formation *in vivo*; furthermore, it significantly improved motor function and reduced the infarct volume in rats with cerebral ischemia[44]. Thus, co-repair of nerve and blood vessel may be more effective[44]. Moreover, the combination of BDNF and NSC transplantation resulted in enhanced therapeutic effects when compared to transplantation of NSCs alone[89]. However, the combination of these cytokines with cells may have some unavoidable problems such as dosage and injection methods; thus, a safe dosage must be first established to avoid harmful side effects, and secondly, a proper balance between cytokines and cells should be determined.

POTENTIAL PROBLEMS WITH NSC TRANSPLANTATION

Although both preclinical and clinical studies have confirmed that transplantation of exogenous NSCs can treat various refractory nervous system diseases such as ischemic stroke and neurodegenerative diseases, there are still some limitations and potential side effects including a large-scale production bottleneck of stem cells, potential allogeneic rejection of cells, risk of cell tumorigenicity, grafting or survival efficiency of cell transplantation, difficulties with the administration route of cells, and targeting problems. All of those limit the rapid clinical transformation and application of NSCs.

The first limitation pertains to the source of NSCs. Given that they only exist in a specific stem cell pool of the brain[22,25], it is difficult to obtain a large number of homogenous cells *in vitro*. Additionally, other limitations in procuring NSC also exist such as the gradual aging of culture cells, repeated extraction and infusion of cells, cost, safety, and ethical issues. Secondly, the obtained exogenous stem cells should be allogeneic, a potential obstacle in the application of SCs is immune-rejection after cell transplantation[90,91]. Similar to any tissue or organ transplantation, allogeneic stem

cells can be rejected by the host immune system. The main reason for this is that the transplanted tissue does not match the HLA in the host. Typically, host T lymphocytes recognize MHC class 1-protein antigens on other cells, if they do not match, the immune system is activated and begins to attack the transplanted cells. Although the brain has historically been considered an immune privileged area, current studies have found that immune cells can also produce immune rejection in the brain[92,93].

Furthermore, the most prominent problem of cell therapy is the potential tumorigenicity of transplanted exogenous stem cells. Stem cells, especially embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, are pluripotent cells that may form teratomas or malignant tumors when implanted into a living host[94,95]. Studies have found that iPS cells derived from B6 mouse embryonic fibroblasts undergo immunological rejection after transplantation into B6 mice and also produce teratomas. ES cells derived from B6 mice also produce teratogenesis in mice, even though no significant immune rejection occurs[95,96]. Simultaneously, the tumorigenicity of iPS cells is also related to the mutagenesis of the c-Myc gene insertion site and the persistent expression of reprogrammed exogenous genes[96-98]. A small number of contaminated exogenous cells may also induce tumorigenesis in allogeneic transplantation, and even very small amounts of contaminating undifferentiated ES cells have been found to produce tumors in nude mice[99]. Therefore, the safety regarding the use of pluripotent cells cannot be ignored. Although NSCs have not yet been found to be tumorigenic, their abnormal proliferation after *in vivo* transplantation may lead to tumor formation.

Finally, a series of cascades following cerebral ischemia, neuro-inflammation, and immune responses can severely affect the survival of cells after transplantation, reduce their ability of replacing damaged neurons, and eliminate the therapeutic effect of cell transplants[80,90,100]. In addition, inflammatory factor could promote glial differentiation of NSCs, resulting in the generation of GFAP-positive cells. And the immune response produced after cell transplantation may also facilitate NSC differentiation into glial cells. This suggests that inflammation may inhibit neuronal generation. However, stem cell-based therapies can modulate the host inflammatory response to recreate a favorable cellular microenvironment and prevent further endogenous cell death. Through regulation of immune inflammation, transplanted cells can increase the chances of endogenous cell survival, but the mechanisms are not clear and need to be further explored.

PROSPECT-STEM CELL-BASED CELL-FREE THERAPY STRATEGY

Currently, stem cell-based therapy is the most promising method for the treatment of refractory diseases. A large number of studies[38,40,48-50] have confirmed the effectiveness and feasibility of stem cell therapies for refractory neurological diseases including ischemic stroke. Although transplanted exogenous NSCs can provide neuroprotective effects after acute stroke and supplement lost nerve tissue for chronic stroke (through direct cell replacement and enhanced endogenous repair), the ultimate goal of complete recovery is not reached, and there are still some problems that need to be resolved.

Several studies[101,102] have confirmed that the paracrine effect plays a major role in stem cell transplantation, and the main molecules responsible for this paracrine effect are extracellular vesicles (EVs). Exosomes are small molecules of exocytic vesicles derived from cells that play a major role in EVs. Exosomes are about 30-150 nm in diameter and can be secreted by all cells including proteins, lipids, and RNAs; furthermore, they play a very important role in cell-to-cell communication[103-105]. Since exosomes, in part, possess active ingredients and functional properties of the cells from which they are derived, they can be used to develop a new type of cell-free treatment. Relevant preclinical and clinical studies[106-108] have confirmed that exosomes derived from stem cells are safe and effective; not only can they cross biological barriers (such as the blood-brain barrier, BBB), but they also exhibit immune tolerance and are relatively stable (no immune rejection) *in vivo*. At the same time, the tumorigenicity of exogenous cells can be avoided (due to the cell-free treatment). In addition, they can be modified by nanomedicine technologies to enhance their therapeutic effects, and facilitate the route of administration or the targeting of treatment[106-108].

Exosomes have been developed as early diagnostic markers for some diseases (such as tumors) and are developing into novel molecules with potential targeted therapeutic effects. Although exosomes were discovered 30 years ago, their clinical

relevance has significantly increased in recent years[107-109]. Furthermore, there is growing evidence demonstrating that exosomes are critical for the benefits of cell therapy[106,110-113]. Since exosomes can overcome many of the risks and difficulties associated with cell therapy, they can be developed as a new strategy to replace stem cell therapy (stem cell-based cell-free treatment method)[101,110,111,114-117]. However, the therapeutic potential and mechanism of exosomes in the nervous system, especially NSC-derived exosomes, has not been studied extensively. For example, the dynamic migration and kinetics of transplanted exosomes derived from NSCs *in vivo*, the type of exosome acting on cells, and the action mode with the local immune micro-environment need to be further studied. Moreover, the specific components of exosomes (such as proteins or miRNAs or lncRNAs) that have a significant potential of action are also unknown and need to be further explored.

In conclusion, currently the most promising treatment approach for refractory neurological diseases including ischemic stroke is based on stem cell transplantation (Figure 3). As a special type of stem cells that are present only in the nervous system, NSCs play a very important role in repairing neurological diseases. NSCs are a type of natural seed resource that not only can supplement necrotic nerve cells or tissues, but also participate in endogenous repair mechanisms. Furthermore, exosomes derived from NSCs have similar functional properties, and may serve as a new research topic in the field of stem cell research. Thus, using stem cell-based cell-free treatment, exosomes can be developed as a new therapeutic strategy, and they may play a more important role in the future.

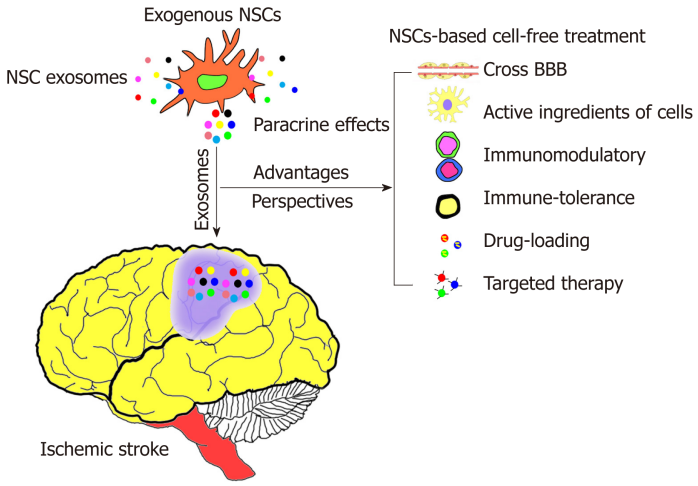

Figure 3 Exosomes derived from neural stem cells are critical for the benefits of stem cell-based therapy. NSCs-exosomes can overcome many of the risks and difficulties associated with stem-cell-based therapy. The exosomes contain the active ingredients of cells, can cross the BBB freely, mediate the immunomodulatory, have no immune tolerance, load drugs and can be labelled for targeted therapy *etc*. Currently NSCs-exosomes as stem cell-free molecules may be the most promising treatment candidate of stem cell-based therapy for ischemic stroke. NSCs: Neural stem cells; BBB: Blood-brain barrier.

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ORIGINAL ARTICLE

Basic Study

Unmodified autologous stem cells at point of care for chronic myocardial infarction

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Abstract

BACKGROUND

Numerous studies investigated cell-based therapies for myocardial infarction (MI). The conflicting results of these studies have established the need for developing innovative approaches for applying cell-based therapy for MI. Experimental studies on animal models demonstrated the potential of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells (UA-ADRCs) for treating acute MI. In contrast, studies on the treatment of chronic MI (CMI; > 4 wk post-MI) with UA-ADRCs have not been published so far. Among several methods for delivering cells to the myocardium, retrograde delivery into a temporarily blocked coronary vein has recently been demonstrated as an effective option.

AIM

To test the hypothesis that in experimentally-induced chronic myocardial

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infarction (CMI; > 4 wk post-MI) in pigs, retrograde delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells (UA-ADRCs) into a temporarily blocked coronary vein improves cardiac function and structure.

METHODS

The left anterior descending (LAD) coronary artery of pigs was blocked for 180 min at time point T0. Then, either 18 × 10^6 UA-ADRCs prepared at "point of care" or saline as control were retrogradely delivered *via* an over-the-wire balloon catheter placed in the temporarily blocked LAD vein 4 wk after T0 (T1). Effects of cells or saline were assessed by cardiac magnetic resonance (CMR) imaging, late gadolinium enhancement CMR imaging, and post mortem histologic analysis 10 wk after T0 (T2).

RESULTS

Unlike the delivery of saline, delivery of UA-ADRCs demonstrated statistically significant improvements in cardiac function and structure at T2 compared to T1 (all values given as mean ± SE): Increased mean LVEF (UA-ADRCs group: 34.3% ± 2.9% at T1 *vs* 40.4 ± 2.6% at T2, $P = 0.037$; saline group: 37.8% ± 2.6% at T1 *vs* 36.2% ± 2.4% at T2, $P > 0.999$), increased mean cardiac output (UA-ADRCs group: 2.7 ± 0.2 L/min at T1 *vs* 3.8 ± 0.2 L/min at T2, $P = 0.002$; saline group: 3.4 ± 0.3 L/min at T1 *vs* 3.6 ± 0.3 L/min at T2, $P = 0.798$), increased mean mass of the left ventricle (UA-ADRCs group: 55.3 ± 5.0 g at T1 *vs* 71.3 ± 4.5 g at T2, $P < 0.001$; saline group: 63.2 ± 3.4 g at T1 *vs* 68.4 ± 4.0 g at T2, $P = 0.321$) and reduced mean relative amount of scar volume of the left ventricular wall (UA-ADRCs group: 20.9% ± 2.3% at T1 *vs* 16.6% ± 1.2% at T2, $P = 0.042$; saline group: 17.6% ± 1.4% at T1 *vs* 22.7% ± 1.8% at T2, $P = 0.022$).

CONCLUSION

Retrograde cell delivery of UA-ADRCs in a porcine model for the study of CMI significantly improved myocardial function, increased myocardial mass and reduced the formation of scar tissue.

Key words: Adipose tissue-derived regenerative cells; Chronic myocardial infarction; Heart failure; Stem cells; Translational medicine; Point of care cell therapy

Core tip: We report results derived from a feasibility study on pigs whose left anterior descending artery was occluded for 180 min. Four weeks later, 18 × 10^6 fresh, uncultured, unmodified, autologous adipose-derived regenerative cells were retrogradely delivered into the balloon-blocked left anterior descending vein (control: delivery of saline). Another 6 wk later, the mean left ventricular mass (+29%; $P < 0.001$) and cardiac output (+37%; $P = 0.002$) had significantly increased after cell delivery. The unique combination of the procedure used for isolating stem cells and the novel cell delivery route applied in the present study potentially opens new horizons for clinical therapy for chronic myocardial infarction.

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INTRODUCTION

Heart failure and myocardial infarction (MI) are consequences of ischemic heart disease (IHD)[1]. In recent years cell-based therapies have emerged as a promising strategy to regenerate ischemic myocardium[2-4]. However, the generally disappointing outcome of related clinical trials established a need for developing novel, more effective cell-based therapies for MI[5]. In this regard, it is of note that the treatment of

chronic MI (*i.e.*, patients with a previous MI) (CMI) requires a different approach than the treatment of acute MI (AMI). Specifically, studies on animal models demonstrated that in AMI, cell-based therapies may primarily act *via* anti-apoptotic and anti-inflammatory mechanisms[6], whereas in CMI there is primarily a need for replacing the, often large, loss of contractile tissue[7]. Using a rat model for the study of MI, it was found that apoptosis of both cardiomyocytes and nonmyocytes mostly takes place during the first 4 wk after MI induction[8]. In addition, a study using a rat model for the study of CMI found that the long-term ability of allogeneic mesenchymal stem cells (MSCs) to preserve function in IHD is limited by an immune response, whereby allogeneic MSCs change from an immunoprivileged to an immunogenic state after differentiation[9]. The latter may have substantially contributed to the relatively poor outcome of a recent clinical trial on CMI treatment with allogeneic adipose-derived stem cells (improvement of the left ventricular ejection fraction (LVEF) from an averaged 28.8% to an averaged 31.7% (on average +2.9% absolute change or +10% relative change) at 6-mo follow-up)[10]. Thus, novel approaches for developing cell-based therapies for CMI should be based on the use of autologous MSCs.

Stem cell density has been reported to be significantly higher in adipose tissue than in bone marrow (5% to 10% *vs* 0.1%)[11]. Moreover, fresh, uncultured, unmodified, autologous adipose-derived regenerative cells (UA-ADRCs) [also called stromal vascular fraction (SVF)] have the advantage over culture-expanded adipose-derived stem cells (ASCs) that UA-ADRCs allow for immediate usage at point of care, combined with low safety concerns, since no culturing or modification is applied.

Several experimental studies on animal models have demonstrated the potential of UA-ADRCs for treating AMI[12-14], and a first clinical trial ("APOLLO") showed promising preliminary results[15]. In contrast, no studies on the treatment of CMI (> 4 wk post-MI) with UA-ADRCs have been published.

Thus, it was the aim of the present feasibility study to test in a porcine model for the study of CMI the following hypotheses: (1) Occlusion of the left anterior descending (LAD) coronary artery for three hours results in a clinically-relevant reduction of the LVEF to less than 40% on average 4 wk post-MI (demonstrating significance of the used animal model); (2) Delivery of UA-ADRCs into the LAD vein 4 wk post-MI in this model leads to improved LVEF by more than 15% (relative change) on average 10 wk post-MI (primary objective of this study); and (3) The same animal model shows improvements in cardiac structure 6 wk after delivery of UA-ADRCs (*i.e.*, 10 wk post-MI) (secondary objective of this study).

This study was performed on a porcine model for the study of CMI (experimentally-induced transmural MI) because (1) the pattern of coronary arteries and distribution of blood supply in the porcine heart is remarkably similar to that in the human heart[16]; (2) unlike rodent and rabbit models, porcine models for the study of CMI allow for the application of UA-ADRCs with the same standard of care and the same instrumentation as in humans[12]; and (3) the therapeutic outcome can be evaluated with MRI. The latter is regarded as the technique that allows the most valid (*i.e.*, accurate and reproducible) and comprehensive measurements of cardiac structure (including chamber dimensions, volumes and infarct size in the case of studies on MI) and related cardiac function (including LVEF, cardiac output, stroke volume, end-diastolic volume and end-systolic volume)[17].

MATERIALS AND METHODS

Ethics statement

All experiments were performed in accordance with the guidelines published by the NIH[18], and under a protocol reviewed and approved by the Institutional Animal Care and Use Committee at Houston Methodist Hospital (Houston, TX, United States) (AUP-0910-0019). All appropriate measures were taken to minimize pain and discomfort.

Animal model

Twenty-five pigs (Yorkshire breed; aged 5-7 mo at the index procedures; K Bar Livestock, Sabinal, TX, United States) were randomly assigned by opening sealed envelopes for treatment with UA-ADRCs (n = 13; group 1) or sham-treatment with saline (n = 12; group 2), respectively (a schematic of the overall study design is illustrated in Figure 1). Eight of these animals could not be included in the final analysis for the following reasons: One animal died during anesthesia before MI induction, four animals died during MI induction, one animal had to be euthanized due to a musculoskeletal injury before the end of the study, in one animal the injection of UA-ADRCs deviated from the protocol, and one animal had a pre-existing

cardiac abnormality. As a result, the final analysis was performed on data from nine animals in group 1 and eight animals in group 2.

Induction of myocardial infarction

Myocardial infarction was induced in all animals from groups 1 and 2 at time point T0. Anti-platelet therapy and (after establishing vessel access) anti-coagulant therapy were performed as previously described[13].

Anti-platelet therapy was administered orally, consisting of 325 mg Acetylsalicylic Acid (Aspirin; Bayer, Leverkusen, Germany) 2 d before T0 (*i.e.* on day T0-2), as well as on T0-1, and Clopidogrel (Plavix; Sanofi-Aventis Pharma, Paris, France) with a loading dose of 300 mg on day T0-2 and 75 mg on days T0-1 and T0). In addition, the animals received the beta blocker Bisoprolol (Concor; Merck, Darmstadt, Germany) from T0-2 to T0+5 (1.25 mg per day orally).

Anti-coagulant therapy was intravenously administered after establishing vessel access as follows: Acetylsalicylic Acid 500 mg (Aspisol; Bayer, Leverkusen, Germany), Enoxaparin 1 mg/kg bolus, then 0.5 mg/kg every 4h (Lovenox; Sanofi-Aventis Pharma), and Eptifibatide prior to balloon occlusion in two 180 μg/kg boluses, 10 min apart, followed by a 2 μg/kg/min infusion during balloon occlusion (Integrilin; SP Europe, Bruxelles, Belgium).

A baseline coronary angiography (CA) (Figure 2A) and a ventriculography in the right and left anterior oblique views (RAO/LAO VG) was performed using an Axiom Artis system (Siemens, Erlangen, Germany). Then, a coronary angioplasty balloon (length 9 mm, diameter 3.0-3.5 mm; Maverick OTW; Boston Scientific, Marlborough, MA, United States) was directed over a 0.014" guide wire (Choice Floppy; Boston Scientific) into the LAD artery, and inflated for three hours at the minimal pressure (typically 2 atm) required for a complete occlusion (Figure 2B). After three hours, the balloon was deflated and a post-MI CA was performed to insure vessel patency (Figure 2C). Catheters and sheaths were removed, and the animal was taken care of as previously described[13]. Enoxaparin 1 mg/kg (Lovenox; Sanofi-Aventis Pharma, Paris, France) was subcutaneously administered at the end of the MI induction procedure.

Isolation of adipose-derived regenerative cells

Cells were isolated during catheterization at 4 wk after MI induction (*i.e.*, at time point T1). Following incision with a scalpel, 12-25 g of subcutaneous adipose tissue was harvested from the nuchal region of each pig. The tissue was divided into aliquots of about 6-10 g each. Then, each aliquot was processed using the Transpose RT system (InGeneron, Houston, TX, United States) for isolating UA-ADRCs from adipose tissue. To this end, each aliquot was minced and incubated together with enzymatic Matrase Reagent (InGeneron) for 1 h under agitation in the processing unit at 39°C, according to the manufacturer's instructions for use. Note that UA-ADRCs that were isolated from adipose tissue with the Transpose RT system and the enzymatic Matrase Reagent were comprehensively characterized in a number of studies[19-21]. This included the demonstrated expression of the regenerative cell-associated genes Oct4, Klf4 and Hes3[21], as well as their differentiative potential into adipogenic, osteogenic, hepatogenic and neurogenic cell lines[21]. On this basis, UA-ADRCs isolated from adipose tissue with the Transpose RT system and the enzymatic Matrase Reagent were used in a number of clinical pilot trials[22-24], and are currently under investigation in a number of Investigational Device Exemption (IDE) studies approved by the United States Food and Drug Administration[25-29].

Characterization of adipose-derived regenerative cells

UA-ADRCs were characterized by cell counting, measuring cell viability, colony-forming unit (CFU-F) assays and flow cytometry.

For counting cells, they were stained with fluorescent nucleic acid stain (SYTO13; Life Technologies, Grand Island, NY, United States) following the manufacturer's instructions, and then counted using a hemocytometer under an Eclipse Ti-E inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) using a PlanFluor 10 × objective [numerical aperture (NA) = 0.3] (Nikon).

The viability of UA-ADRCs was determined by preparing a 3:1 dilution of the cell suspension in 0.4% Trypan Blue solution. Nonviable cells were counted using a hemocytometer under the same microscope, and were correlated to the number of viable nucleated cells.

The CFU-F assay was implemented as an indicator of stemness[30]. To this end, freshly isolated nucleated cells from each preparation of UA-ADRCs were plated at a density of 100,000 cells per 35-mm-diameter well in triplicate, and incubated for 14 d in standard growth media as previously described[31]. Media were changed twice weekly. Afterwards, the cells were fixed with 4% formalin, stained with hematoxylin for 10 min and washed with phosphate-buffered saline (PBS). Photomicrographs were

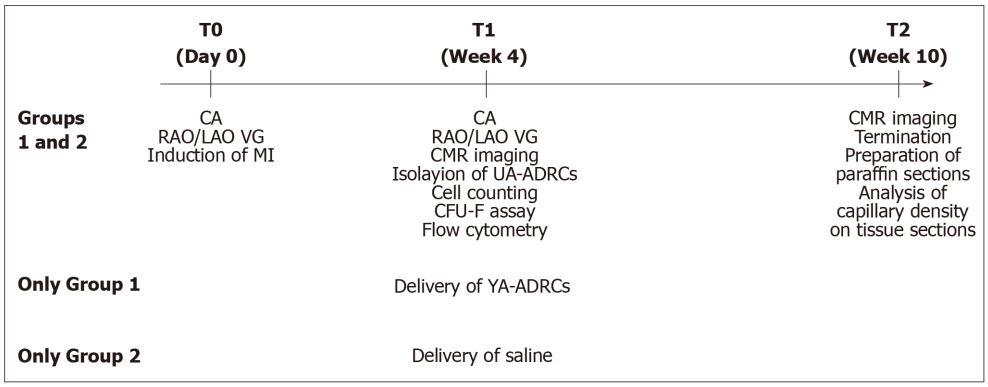

Figure 1 Experimental details of the present study. CA: Coronary angiography; RAO/LAO VG: Ventriculography in right and left anterior oblique views; MI: Myocardial infarction; CMR: Cardiac magnetic resonance; UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

taken from five randomly chosen fields-of-view per well with a DS-Fi1 CCD color camera (2560 x 1920 pixels) (Nikon) attached to an Eclipse Ti-E inverted microscope (Nikon) and NIS-Elements AR software (version 4.13) (Nikon), using a Plan EPI 2.5× objective (NA = 0.075) (Nikon). A colony-forming unit was defined as a cluster containing at least ten fibroblast-like fusiform cells[20]. Two experienced investigators individually counted the colonies.

For flow cytometric analysis, UA-ADRCs were cultured for 24 h, incubated with antibodies for 30 min, washed, re-suspended in 1 mL PBS with 10% fetal bovine serum and 1% sodium azide, and directly analyzed by flow cytometry employing a BD FACSAria Fusion device (BD Bioscience, San Jose, CA, United States). Antibodies against porcine CD29 (antibodies-online, Aachen, Germany), CD44 (Abcam, Cambridge, MA, United States), NG2 (Abcam), Oct4 (Novus Biologicals, Littleton, CO, United States), CD31 (Gentex, Irvine, CA, United States), CD45 (Abcam), Nestin (Santa Cruz Biotechnology, Dallas, TX, United States), CD146 (Gentex, Irvine, CA, United States) and CD117 (eBioscience, San Diego, CA, United States) were used.

Delivery of cells or saline as controls

Four weeks after MI induction, a 6F Amplatz right 1 guide catheter (Mach 1; Boston Scientific) was advanced over a 0.035'' wire (J-tip Starter; Boston Scientific) through the jugular vein into the coronary sinus (CS). Once in place in the CS, an angioplasty balloon (length 8-12 mm; diameter 3.0-3.5 mm; Maverick OTW; Boston Scientific) was positioned over a 0.014'' guide wire (Choice Floppy; Boston Scientific) at the site of the LAD vein that corresponded to the previous LAD artery occlusion site (Figure 2D). After the wire was removed, either a suspension of UA-ADRCs (18×10^6 cells in 10 mL saline; group 1) or 10 mL saline alone (group 2), respectively, were delivered at a rate of approximately 0.25 mL/s through the catheter's central lumen retrogradely into the LAD vein. The angioplasty balloon was kept inflated during the entire delivery procedure, and for five minutes after the delivery. Operators were blinded to the group assignment, *i.e.*, they did not know whether UA-ADRCs or saline was delivered.

Cardiac magnetic resonance (CMR) imaging

All animals underwent CMR)imaging directly before the delivery of UA-ADRCs or saline at T1, as well as 6 wk after T1 (*i.e.* at T2). The scans were performed using a 1.5 Tesla MRI scanner (Avanto; Siemens).

Steady-state free precession (SSFP) CMR cine images were acquired using an electrocardiogram-gated SSFP pulse sequence in multiple short-axis and long-axis views. Short-axis views were obtained every 1 cm from the atrioventricular ring to the apex to cover the entire left ventricle (slice thickness 6 mm, inter-slice gap 4 mm, echo time (TE) 1-1.5 ms, temporal resolution (TR) 35–45 ms, flip angle 50-90°).

Late gadolinium enhancement (LGE) CMR imaging was performed approximately ten minutes after the administration of 0.15 to 0.20 mmol/kg of gadolinium (Magnevist; Bayer Inc. Mississauga, ON, Canada) in slice orientations identical to CMR cine imaging using a standard inversion recovery gradient echo pulse sequence. Manual adjustment of the time from inversion (TI) was performed in all cases in order to achieve the nulling of normal viable myocardium[32]. Typical imaging parameters were matrix 256 × 192, slice thickness 6 mm, gap 4 mm, TI 250–350 ms, TE 2.0–2.5 ms, flip angle 30°, and parallel imaging with a two-fold acceleration factor.

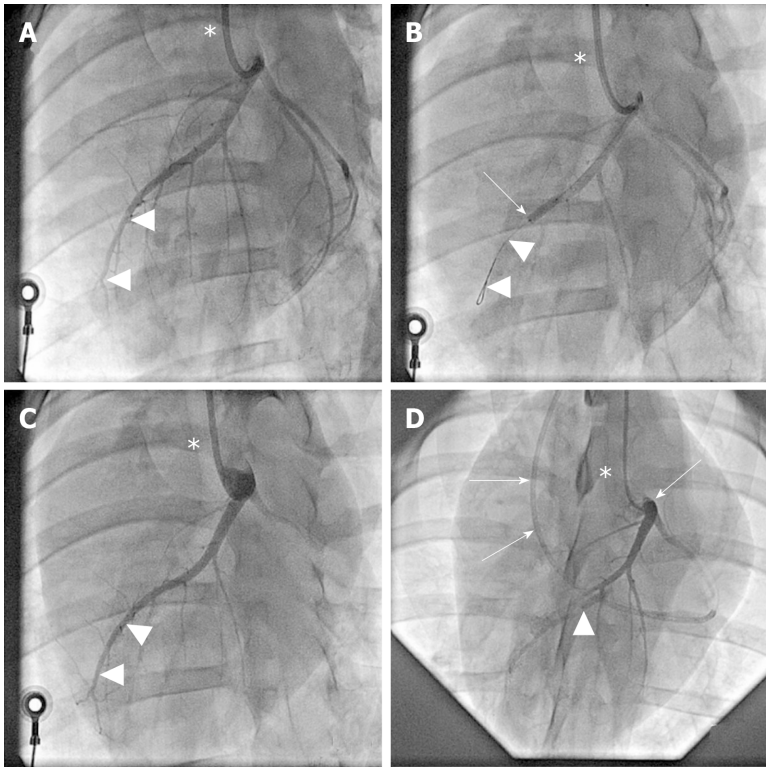

Figure 2 Angiographic details of the present study. A: Baseline coronary angiography of a porcine heart in a left anterior oblique view (in all panels, the white asterisk indicates the angiography catheter positioned in the left main coronary ostium). The white arrowheads indicate the distal LAD artery; B: Induction of myocardial infarction by occlusion of the LAD artery for three hours through an inflated balloon catheter at time point T0. The white arrow indicates the position of the inflated balloon inside the mid LAD artery, whereas the white arrowheads show the guidewire in the distally occluded LAD artery; C: Complete reperfusion of the LAD artery (white arrowheads) three hours after removal of the balloon occlusion; D: Delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells (or saline as control, respectively) through the LAD vein (matching the initial LAD artery occlusion site) into the infarction area 4 wk later (*i.e.* at time point T1). To this end, the LAD vein was occluded with an inflated "over the wire" balloon catheter advanced through a guiding catheter (black arrows), placed from the right jugular vein into the right atrium and then into the coronary sinus. The inflated balloon (filled with contrast dye; white arrowhead) in the coronary LAD vein had the aim to prevent the backflow of cells when they were delivered through the distal orifice of the central lumen of this balloon catheter. LAD: Left anterior descending.

Analysis of CMR images

Image analysis was performed with cvi[42] software (Circle Cardiovascular Imaging Inc., Calgary, AB, Canada). Endocardial and epicardial borders were traced using planimetry on stacks of short-axis cine images in end diastole and end systole (Figure 3). Based on these data, the following variables were calculated for each animal: LVEF, cardiac output, stroke volume, end-diastolic volume (EDV), end-systolic volume (ESV) and left ventricular mass. LVEF was calculated as the difference between EDV and ESV, divided by EDV. The left ventricular mass was calculated by the difference between the left ventricular epicardial and endocardial volumes during end systole, multiplied by myocardial density (1.05 g/cm^3)[33]. For quantification of scar tissue, a region of normal myocardium was independently chosen on each short axis image showing the left ventricle, and hyper-enhanced regions in each slice were located. Hyper-enhancement was identified as areas of signal intensity ≥ 5 standard deviations greater than normal myocardium[34]. Two well-trained MRI physicians, blinded to the group assignment, evaluated the selected regions and graded the findings in all segments on each short axis image.

Analysis of regional replacement fibrosis

This analysis was performed with the cvi[42]® software (Circle Cardiovascular Imaging Inc.) on the LGE CMR images as follows: (1) The left ventricle was divided into 17 segments, as recommended by the American Heart Association (AHA)[35]. With the exception of the apical segment (No. 17), which cannot be investigated on the short axis, transversal images through all segments of the mid left ventricle of a porcine heart that were applied in this study (Figure 3) were analyzed, yielding a total of 9×16 (group 1) + 8×16 (group 2) = 272 analyzed segments; (2) The signal intensity (SI) of a

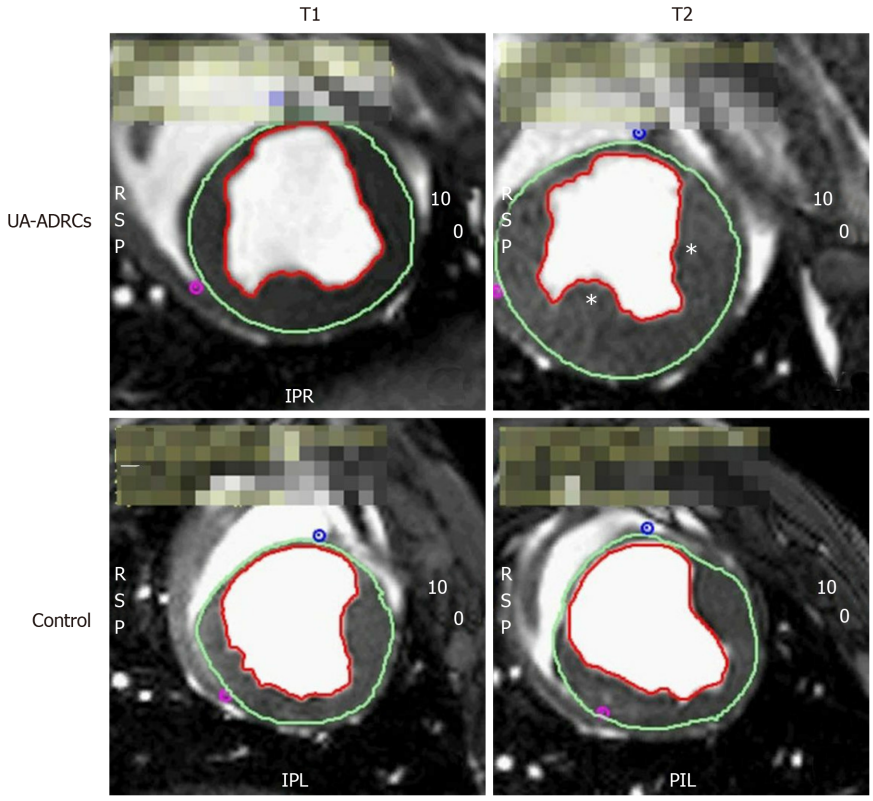

Figure 3 Steady-state free precession CMR imaging of the porcine heart. A-D: Representative examples of end-systolic, short axis, transversal images through the mid left ventricle of a porcine heart obtained with SSFP CMR imaging for analyzing hemodynamic parameters and wall motility at time points T1 (A, C) and T2 (B, D) of a representative animal in group 1 (delivery of UA-ADRCs) (A, B) and a representative animal in group 2 (control) (C, D) (details are provided in the main text). In all panels, the epicardial contours are highlighted in green, and the endocardial contours in red. Note the increased end-systolic thickness of the left ventricular wall at T2 after delivery of UA-ADRCs at T1 (asterisks in B) compared to the delivery of saline at T1 (D). In the examples presented here, the left ventricular ejection fraction was 27.2% in (A), 39.7% in (B), 22.5% in (C), and 27.2% in (D). CMR: Cardiac magnetic resonance; SSFP CMR: Steady-state free precession cardiac magnetic resonance; UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

given pixel was considered hyper-enhanced when its SI was ≥ 5 standard deviations greater than the SI of normal myocardium (SI > 5 SD); otherwise, its SI was considered normal[34]; (3) For each segment, the relative number of pixels with hyper-enhanced SI was calculated ($NPrel_{SI>5SD}$). These calculations were separately performed for T1 and T2, resulting in segment-specific data $NPrel_{SI>5SD}$-T1 and $NPrel_{SI>5SD}$-T2; and (4) For all segments of a given animal, the difference between $NPrel_{SI>5SD}$-T1 and $NPrel_{SI>5SD}$-T2 was calculated as $\Delta NPrel_{SI>5SD}$ = $NPrel_{SI>5SD}$-T2 - $NPrel_{SI>5SD}$-T1.

Termination

Animals were euthanized according to the Houston Methodist Research Institute Euthanasia for Large Animals Procedure (Houston, TX, United States) after performing CA, RAO/LAO VG and CMR imaging at T2. Intravenous injection of 0.25 mg/kg Pentobarbital/Phenytoin combination was performed in conjunction with isoflurane overdose. Death was verified by the absence of vital signs. Hearts and organs were removed for further histologic and immunofluorescence analysis. Animal carcasses were disposed of in accordance with standard operating procedures of the Houston Methodist Research Institute (Houston, TX, United States).

Histologic processing of heart tissue

Hearts were harvested and fixed in 5% paraformaldehyde. The left ventricle was cut into six transversal, 1 cm-thick slices from apex to base. Then, from each heart, several approximately 1 cm × 1 cm × 1 cm large tissue samples were collected, representing the left ventricular border zone of MI, the core region of MI, and regions of viable myocardium. Specimens were paraffin-embedded and cut into 5 μm-thick tissue sections that were mounted on glass slides and stained with Masson's Trichrome staining, or processed with fluorescence immunohistochemistry.

Fluorescence immunohistochemistry was performed on de-paraffinized and rehydrated sections that were washed with PBS containing 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO, United States) and blocked with 10% casein solution (Vector Laboratories, Burlingame, CA, United States) for 30 min at room temperature. Then, sections were incubated overnight with diluted Rabbit anti-von Willebrand factor (vWF) primary antibody (Abcam) or diluted Rabbit anti-adiponectin (Cy5 conjugated) primary antibody (Biorbyt, San Francisco, CA, United States), and subsequently for 1 h with diluted Goat anti rabbit-IgG secondary antibody (Cy5 conjugated) (Thermo Scientific, Waltham, MA, United States). Counterstaining of nuclei and mounting were performed with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories).

Analysis of microvessel density

The microvessel density was determined on up to four representative sections from each animal showing the left ventricular border zone of MI. Only sections that showed at least 30% of both scar tissue and viable myocardium were considered in this analysis. To this end, photomicrographs covering the entire section were taken with a DS-Fi1 CCD color camera (Nikon) attached to an Eclipse Ti-E inverted microscope (Nikon) using a PlanApo 20× objective (NA = 0.75) (Nikon). Images were analyzed using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, United States). Using a defined grid of ten fields per section with an area of 0.3 mm^2 per field, two independent, blinded evaluators determined the number of microvessels per field (microvessels with a diameter between 2 and 10 μm were counted). Microvessel density was calculated based on microvessel counts on a total of 202 fields (animals in group 1) or 247 fields (animals in group 2), respectively.

Photography

The photomicrographs shown in Figures 4-6 were produced by digital photography using a DS-Fi1 CCD color camera (2560 x 1920 pixels; Nikon) (Figures 4 and 5) or a CoolSNAP HQ2 CCD monochrome camera (1392 x 1040 pixels; Photometrics, Tucson, AZ, United States) (Figure 6) attached to an Eclipse Ti-E inverted microscope (Nikon) and NIS-Elements AR software (Nikon), using the following objectives (all from Nikon): PlanFluor 4 × (NA = 0.13), 10× (NA = 0.3) and 20× (NA = 0.45). The final figures were constructed using Corel Photo-Paint X7 and Corel Draw X7 (both versions 17.5.0.907; Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made using Corel Photo-Paint, without altering the appearance of the original materials.

Statistical analysis

For each combination of animal groups [group 1 (delivery of UA-ADRCS) and group 2 (control)], time point [T1 (4 wk after MI induction) and T2 (10 wk after MI induction)], the mean ± SE were calculated for the following variables: LVEF, cardiac output, stroke volume, EDV, ESV, heart rate, left ventricular mass and relative amount of scar tissue. Group-specific means and SEM were also calculated for the body weight of the animals (determined at T0, T1 and T2) and the microvessel density (investigated only at T2). These calculations were performed with previous testing with the Shapiro-Wilk normality test of whether the values came from a Gaussian distribution. Except for the microvessel density, comparisons between groups were performed with repeated measures two-way analysis of variance, followed by post hoc Bonferroni tests for pairwise comparisons. For microvessel density, comparisons between groups were performed with the unpaired two-tailed Student's *t*-test.

Mean and SEM were also calculated for the variable $\Delta NPrel_{SI>5SD}$ (results of the regional replacement fibrosis analysis). This was separately undertaken for each combination of animal groups (groups 1 and 2) and segments (those that are assigned to the territory of the LAD artery in the human heart, and those that are assigned to the territory of the right coronary artery and the left circumflex coronary artery in the human heart). Comparisons between groups were performed with two-way analysis of variance, followed by post hoc Bonferroni tests for pairwise comparisons.

In all analyses, an effect was considered statistically significant if its associated p value was smaller than 0.05. Calculations were performed using GraphPad Prism (version 7.0 for Windows, GraphPad software, San Diego, CA, United States).

RESULTS

Characterization of UA-ADRCs

The amount of adipose tissue that was obtained from the nuchal region per pig varied between 12-25 g (mean ± SE: 18.1 ± 1.61 g). On average, $0.98 \times 10^6 \pm 0.10 \times 10^6$

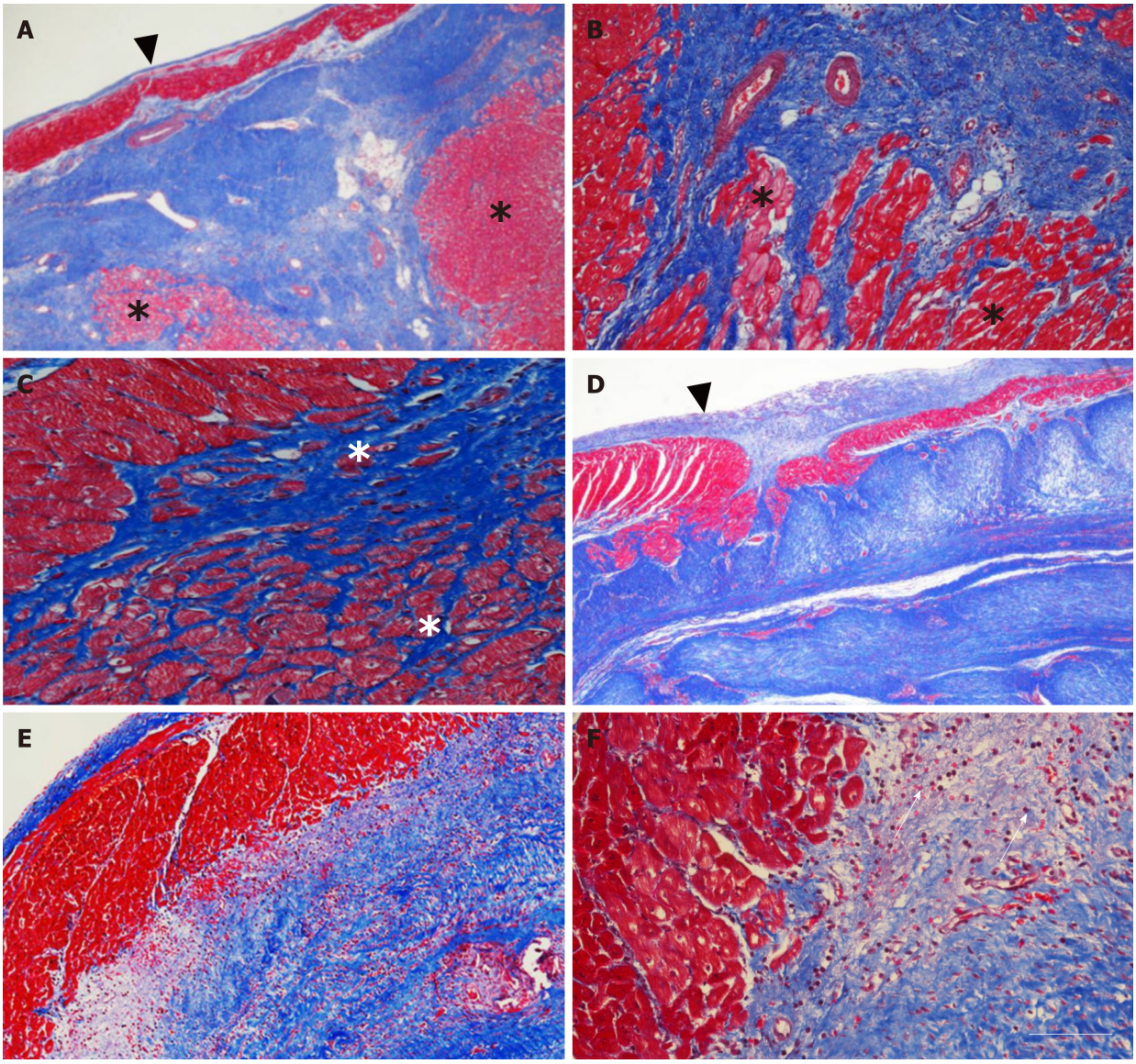

Figure 4 Microstructure of cardiac tissue after delivery of UA-ADRCs or saline. A-F: Representative photomicrographs of paraffin-embedded, 5 μm thick tissue sections stained with Masson's Trichrome staining of post mortem hearts from pigs in group 1 (delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells) (A-C) and group 2 (delivery of saline as control) (D-F) at T2. The arrowheads in (A, D) point to the endocardium, the asterisks in (A-C) indicate patchy islets of cardiomyocytes located within areas of fibrous tissue, and the arrows in (F) point to an infiltration with inflammatory cells. The scale bar in (F) represents 500 μm in (A, D), 200 μm in (B, E), and 100 μm in (C, F). UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

nucleated cells were isolated from each gram of adipose tissue (cell yield), from which 93.3% ± 0.4% cells were viable (live cell yield). The CFU-F value was 11.3%. Flow cytometric analysis demonstrated the following relative numbers of cells immunopositive for a certain marker (sorted in descending order; an example of the results of flow cytometric analysis is depicted in Figure 7): CD29: Between 44.1-52.9% (mean: 48.5%); CD44: Between 34.2-39.8% (mean: 37.0%); CD31: Between 6.0-16.7% (mean: 11.4%); NG2: Between 7.3-12.3% (mean: 9.8%); CD45: Between 3.9-13.9% (mean: 8.9%); Oct4: Between 2.4-6.2% (mean: 4.3%); Nestin: Between 1.9-6.2% (mean: 4.1%); CD146: Between 0.4-0.7% (mean: 0.6%); and CD117: Between 0.1-0.4% (mean: 0.3%).

Improvement of cardiac function after delivery of UA-ADRCs

The following, statistically significant improvements in cardiac function at T2 compared to T1 were found for the animals in group 1 (delivery of UA-ADRCs) but not for the animals in group 2 (control): Increased mean left ventricular ejection fraction (+18%; $P = 0.037$) (primary objective) (Figure 8A and B; mean and SEM data as well as P-values of repeated measures two-way analysis of variance are provided in Table 1), increased mean cardiac output (+37%; $P = 0.002$) (Figure 8C and D, as well as Table 1), and increased mean stroke volume (+41%; $P < 0.001$) (Figure 8E and 8F, as

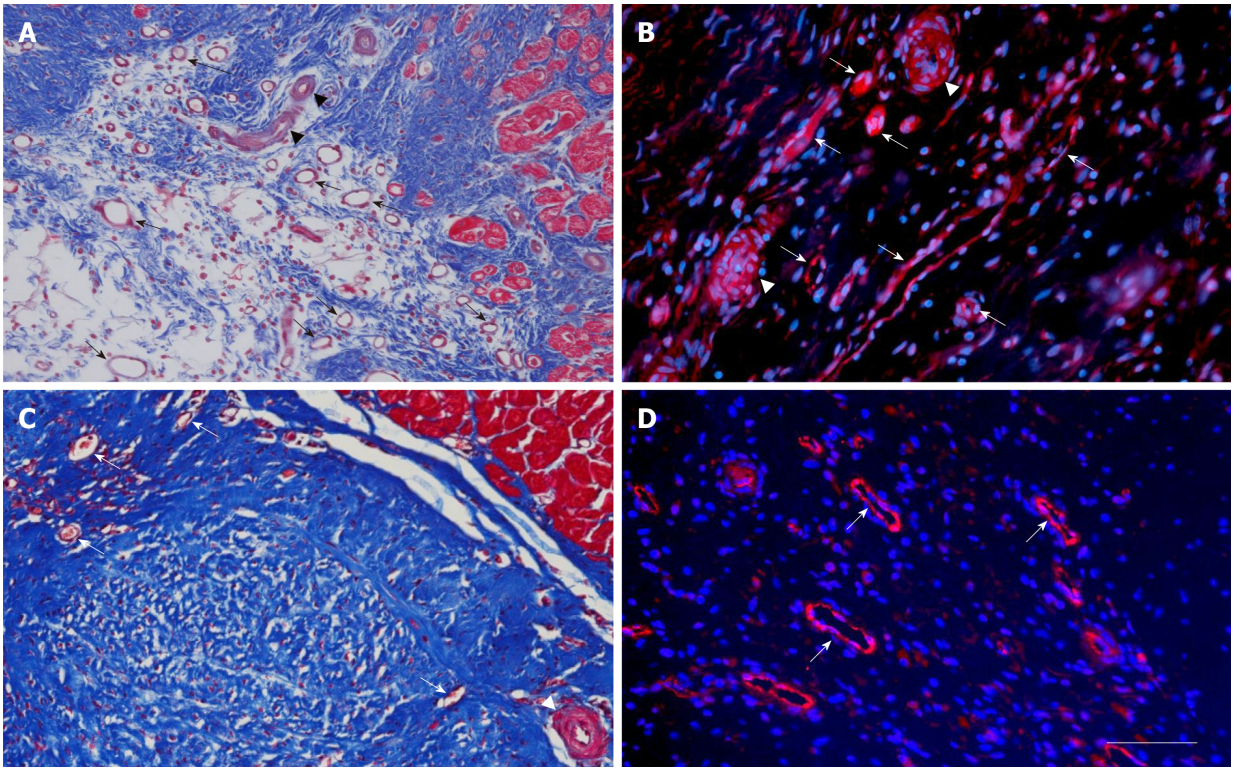

Figure 5 Microvessel density after delivery of UA-ADRCs or saline. The panels show representative photomicrographs of paraffin-embedded, 5 μm thick tissue sections of post mortem hearts from pigs in group 1 (delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells) (A, B) and group 2 (delivery of saline as control) (C, D) at T2. In (A, C), tissue sections were stained with Masson's Trichrome staining. In (B, D), tissue sections were processed with fluorescence immunohistochemistry in order to detect von Willebrand factor (red) (counterstaining with DAPI in blue). The arrows point to microvessels, and the arrowheads to small arterioles. The scale bar shown in D represents 100 μm in (A, C) and 35 μm in (B, D). UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

well as Table 1).

As animals in both groups grew from T1 to T2 (Figure 9), the mean end-diastolic volume significantly increased in both groups from T1 to T2 [group 1: +20% (P < 0.001); group 2: +15% (P = 0.004)] (Figure 8G and H, as well as Table 1). However, the mean end-systolic volume significantly increased only in group 2 (+17%; P = 0.018) but not in group 1 (+9%; P = 0.222) (Figure 8I and J, as well as Table 1), causing a significant increase in mean stroke volume in group 1 (+41%; P < 0.001) but not in group 2 (+10%; P = 0.552). The mean heart rate showed no significant difference between T1 and T2 in both groups (Figure 8K and L, as well as Table 1), causing a significant increase in mean cardiac output only in group 1 (+37%; P = 0.002) but not in group 2 (+7%; P = 0.798).

Improvement of cardiac structure after delivery of UA-ADRCs

For animals in group 1 (delivery of UA-ADRCs) but not in group 2 (control), a significantly increased mean mass of the left ventricle (+29%; P < 0.001) was found at T2 compared to T1 (Figure 10A and C; results of statistical analysis are summarized in Table 1). Furthermore, animals in group 1 (delivery of UA-ADRCs) showed a significantly decreased mean relative amount of scar volume of the left ventricular wall (-21%; P = 0.042) at T2 compared to T1 (Figure 10B and D, Figure 11 and Table 1). Of note, the opposite was observed for animals in group 2 (control), *i.e.*, a significant increase in the mean relative amount of scar volume of the left ventricular wall (+29%; P = 0.022) at T2 compared to T1 (Figure 10B and D, Figure 11 and Table 1).

The regional replacement fibrosis data were visualized using color-coded, AHA 17-segment bullseye plots (Figure 12). Statistical analysis demonstrated significant improvement in LAD segments – but not in non-LAD segments – after delivery of UA-ADRCs (P = 0.008) (results of repeated measures two-way analysis of variance: $P_{Interaction}$ = 0.125; $P_{Territory}$ = 0.936; $P_{Treatment}$ < 0.001) (Figure 13).

Histologic examination demonstrated patchy islets of cardiomyocytes located within areas of fibrous tissue in the left ventricular border zone of the MI in post-mortem hearts of animals in group 1 (delivery of UA-ADRCs) (Figure 4A, 4B and 4C). In contrast, the post mortem hearts of animals in group 2 (control) showed infiltration with inflammatory cells in the same region (Figure 4D, 4E and 4F).

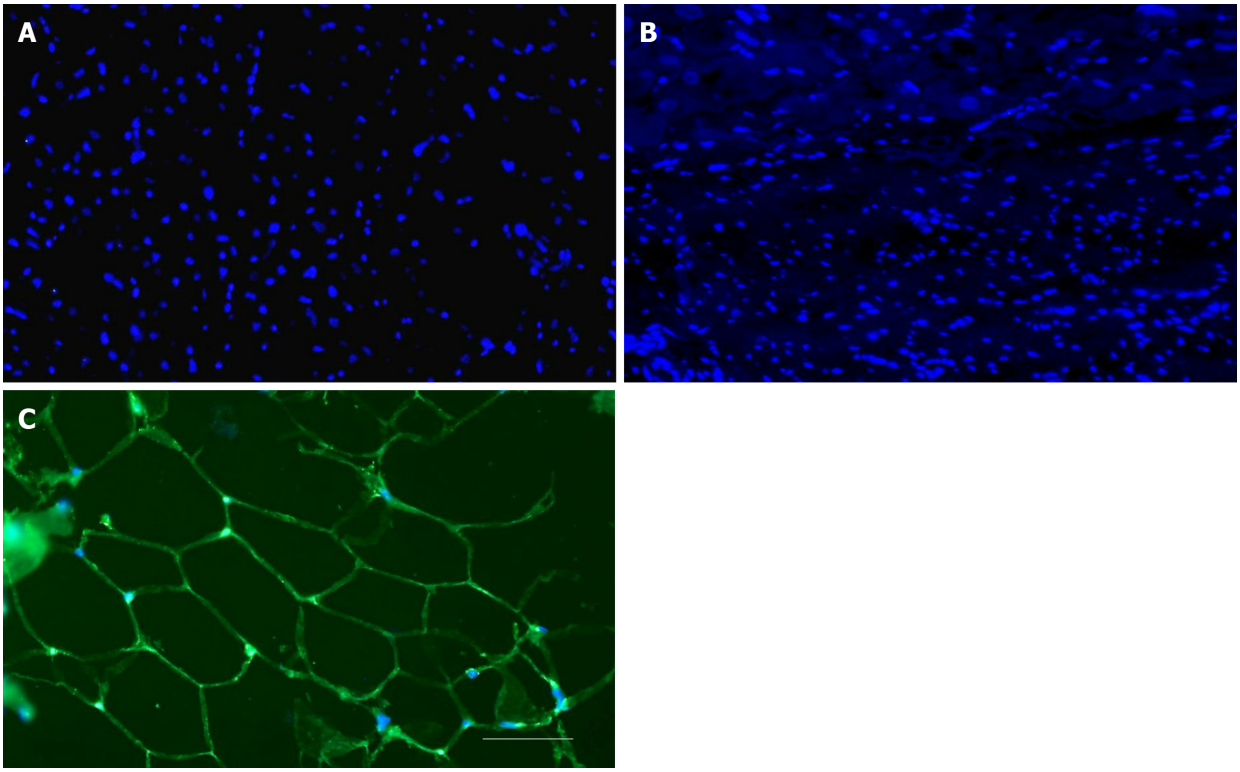

Figure 6 No differentiation of stem cells into adipocytes after delivery of UA-ADRCs. A, B: Representative photomicrographs of paraffin-embedded, 5 μm thick tissue sections of post mortem hearts from pigs in group 1 (delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells) (A) and group 2 (delivery of saline as control) (B), taken from the left ventricular border zone of myocardial infarction at 10 wk. (C) Representative photomicrograph of a paraffin-embedded, 5 μm thick tissue section of subcutaneous adipose tissue from a pig. The sections were stained with DAPI (blue), and processed for immunofluorescent detection of adiponectin (green). The scale bar represents 100 μm. UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

Improvement of cardiac revascularization after delivery of UA-ADRCs

The left ventricular border zone of the MI of the post-mortem hearts in group 1 animals (delivery of UA-ADRCs) exhibited a significant, more than two-fold higher mean microvessel density than the corresponding region in the post-mortem hearts of the group 2 animals (control) at T2 [54.4 ± 3.7 *vs* 26.1 ± 2.8 (mean ± SE) capillaries per mm^2; $P < 0.001$] (Figure 5A and C). Immunofluorescent detection of von Willebrand factor supported this finding (Figure 5B and D).

No differentiation of stem cells into adipocytes after delivery of UA-ADRCs

Cells in the left ventricular border zone of the MI of the post-mortem hearts from the group 1 (delivery of UA-ADRCs) and 2 (control) animals displayed no expression of adiponectin at T2 (Figure 6).

DISCUSSION

This is the first study in which all of the following findings from previous research on cell-based therapies for CMI with high clinical relevance were considered and combined into a single model: (1) Application of the same standard of care and instrumentation as in humans by using a porcine model for the study of CMI (which is not possible when investigating mouse or rat models for the study of CMI); (2) A low baseline LVEF after induction of MI of approximately 35% (according to the guidelines for the diagnosis and treatment of acute and chronic heart failure published by the European Society of Cardiology in 2016[36] (a LVEF ≥ 50% is considered normal or preserved, whereas a LVEF < 40% is considered reduced) (the mean LVEF of healthy landrace pigs with an average body weight of approximately 53 kg is approximately 53%[37]); (3) The use of well-characterized, fresh, uncultured, unmodified autologous adipose-derived regenerative cells prepared at "point of care" (rather than employing cultured and/or modified, autologous or non-autologous cells); and (4) The evaluation of therapeutic success using CMR imaging (rather than using other methods such as echocardiography). Besides this, the UA-ADRCs were retrogradely delivered into the temporarily blocked LAD vein (rather than

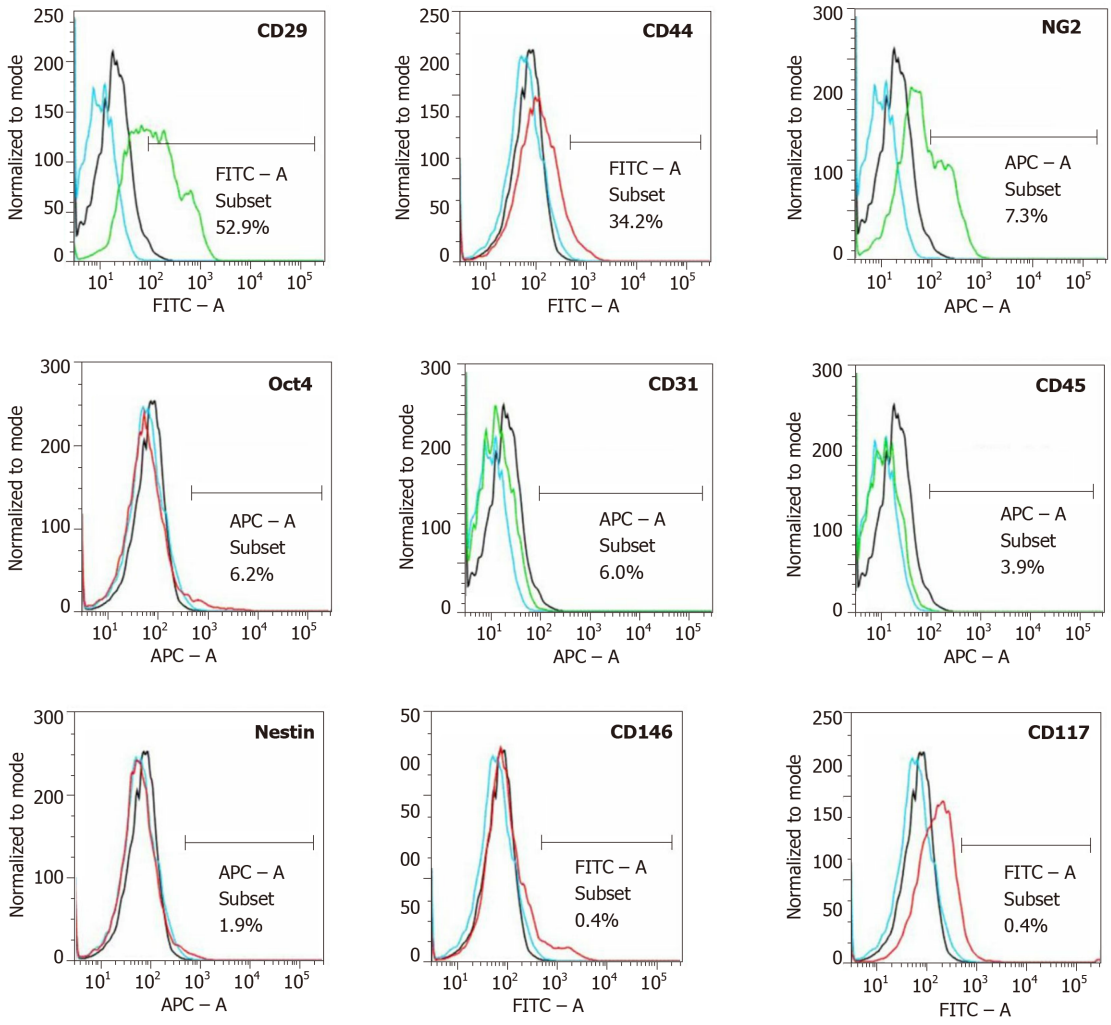

Figure 7 Analysis of cell surface markers of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells from an animal in group 1 using flow cytometry. The cells were stained with monoclonal antibodies for CD29, CD44, NG2, Oct4, CD31, CD45, Nestin, CD146 and CD117 at passage 0. Flow cytometric histographs are representative of triplicate experiments.

transendocardial, intramyocardial or intracoronary cell delivery).

Figure 14 summarizes the key findings [changes in mean LVEF and mean relative amount of scar volume of the left ventricular wall (ScVol) from baseline (*i.e.*, immediately before delivery of cells or control treatment, respectively) to follow-up] from the present study and from all studies on cell therapy for CMI using porcine models that were published so far[38-42] (details of these studies are provided in Table 2). The animals had on average the lowest mean LVEF and the highest mean ScVol at baseline in our study, indicating high clinical relevance. In fact, four[39-42] out of the six studies summarized in Figure 14 investigated porcine models for the study of CMI with moderately reduced LVEF according to a previous study[36], whereas an earlier study by Johnston *et al*[38] as well as ours investigated porcine models for the study of CMI with reduced LVEF[36]. Furthermore, the animals showed the highest increase in mean LVEF and the second-highest reduction in mean ScVol in our study. The mean ScVol was reduced from 19.2% at baseline to 14.2% 8 wk later[38], compared to 20.9% at baseline and 16.6% 6 wk later in our study. Dissimilar to our study, however, no improvement of mean LVEF was found in a former study[38] (37.8% at baseline *vs* 37.6% 8 wk later, compared to 34.3% at baseline *vs* 40.4% 6 wk later in our study).

Analysis of regional replacement fibrosis demonstrated that the reduction in ScVol observed in our study predominantly took place in those left ventricular segments that are assigned to the vascular territory of the LAD in the human heart. Considering that the pattern of coronary arteries and distribution of blood supply in the porcine heart is remarkably similar to that in the human heart[16], these regional replacement fibrosis data support the effectiveness of our therapeutic approach. As indicated in Figure 14, the differences in the outcome between our study and other published studies on cell therapy for CMI using porcine models[38-42] cannot be explained by substantial differences in the time between MI induction and the delivery of cells, or

Table 1 Results of statistical analysis

Parameter	Group 1 (delivery of UA-ADRCs)				Group 2 (delivery of saline as control)				*P* values of repeated measures two-way analysis of variance			
	T1		T2		T1		T2					
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	I	Time	Tr	SM
LVEF	343	289	404	264	378	257	362	245	0.036	0.207	0.912	0.006
CO	273	022	378	023	339	030	363	032	0.049	0.004	0.458	0.019
SV	314	202	443	239	346	299	380	343	0.036	0.001	0.638	0.048
EDV	936	507	1119	624	926	645	1061	717	0.358	< 0.001	0.696	< 0.001
ESV	621	528	676	649	580	524	681	603	0.327	0.005	0.821	< 0.001
HR	870	293	854	419	974	396	955	361	0.940	0.400	0.051	0001
M_{LV}	553	499	713	449	632	335	684	397	0.042	0.001	0.665	0.001
ScVol	209	229	166	119	176	138	227	176	0.002	0.739	0.509	0.019

The time points T1 and T2 are explained in detail in the main text. *P* values < 0.05 are given boldface. T1: Delivery of UA-ADRCs (group 1) or saline (group 2) 4 wk after induction of myocardial infarction; T2, 6 wk after T1 (*i.e.* 10 wk after induction of myocardial infarction); LVEF: Left ventricular ejection fraction (%) (primary objective); CO: Cardiac output (L/min); SV: Stroke volume (mL); EDV: End diastolic volume (mL); ESV: End systolic volume (mL); HR: Heart rate (min^{-1}); M_{LV}: Left ventricular mass (g); ScVol: Relative amount of scar volume of the left ventricular wall (%); I: Interaction; Tr: Treatment; SM: Subject matching; UA-ADRCs: Fresh, uncultured, unmodified autologous adipose-derived regenerative cells.

the time between the delivery of cells and follow-up. Rather, the differences in outcomes were most probably caused by differences in the type of cells delivered and the delivery route. In fact, in all other studies listed in Figure 14[38-42], allogeneic cells were used [cardiosphere-derived cells (CDCs) in[38-40], adipose-derived stem cells (ASCs) in[41], and a combination of bone marrow-derived stem cells (BMSCs) and allogeneic, c-Kit-positive cardiac stem cells (CSCs)[42]]. Allogeneic ASCs and BMSCs indicated benefits in the treatment of porcine and sheep models for the study of acute MI (delivery of cells immediately after[43-45] or within the first 8 d[46] after induction of MI). However, both the aforementioned studies employing porcine models for the study of CMI[38-42] as well as a recent clinical trial on treatment of CMI with allogeneic ASCs[10] revealed relatively poor outcomes (on average between -0.5% and +1.9% absolute change or between -1.0% and +4.6% relative change in mean LVEF at follow-up in the studies on porcine models[38-42], and on average +2.9% absolute change or +10% relative change in mean LVEF at 6-mo follow-up in the clinical trial[10]). This is significantly less than the average +6.1% absolute change or +17.9% relative change in the mean LVEF reported in the present study. As previously mentioned above, the long-term ability of allogeneic stem cells to preserve function in the treatment of CMI may be limited by an immune response, whereby the allogeneic cells change from an immunoprivileged to an immunogenic state after differentiation[9]. In fact, 40% of the IHD patients enrolled in the clinical trial on treatment of CMI with allogeneic ASCs[10] developed donor-specific *de novo* human leukocyte antigen (HLA) class I antibodies, and 20% of the IHD patients already had donor-specific HLA antibodies at baseline[10]. Using porcine models, Tseliou *et al*[40] found serum alloantibodies only in one out of eight animals 4 wk after delivery of allogeneic CDCs, whereas the other studies[38,39,41,42] did not investigate the development of serum alloantibodies. Natsumeda *et al*[42] occasionally detected inflammatory cells in myocardial tissue 3 mo after delivery of allogeneic BMSCs and CSCs, but attributed this finding to CMI. Blásquez *et al*[39] investigated various immunological parameters 4 wk after the delivery of allogeneic CDCs, and concluded that the observed changes could exert a modulation in the inflammatory environment of the heart in CMI, indirectly benefiting endogenous cardiac repair. However, because of the lack of a control group in the study by Blásquez *et al*[39], this conclusion might be treated with caution.

Nonetheless, it would not be correct to conclude that therapies based on allogeneic cells are in principle less efficient than therapies based on autologous cells for treatment of chronic cardiac failure. Rather, a recent randomized clinical trial (RCT) on patients who suffered from chronic nonischemic dilated cardiomyopathy (NIDCM) who averaged more than 6 years with mean LVEF of approximately 26% at baseline (POSEIDON-DCM)[47] reported improvement of LVEF by averaged +8.0% absolute change 1 year after transendocardial delivery of 100×10^6 allogeneic BMSCs (P = 0.004 compared to baseline), compared to improvement of LVEF who averaged +5.4% absolute change 1 year after transendocardial delivery of 100×10^6 autologous BMSCs (P = 0.116 compared to baseline). The authors of the POSEIDON-DCM RCT[47] hypothesized that the following reasons could explain the better outcome after

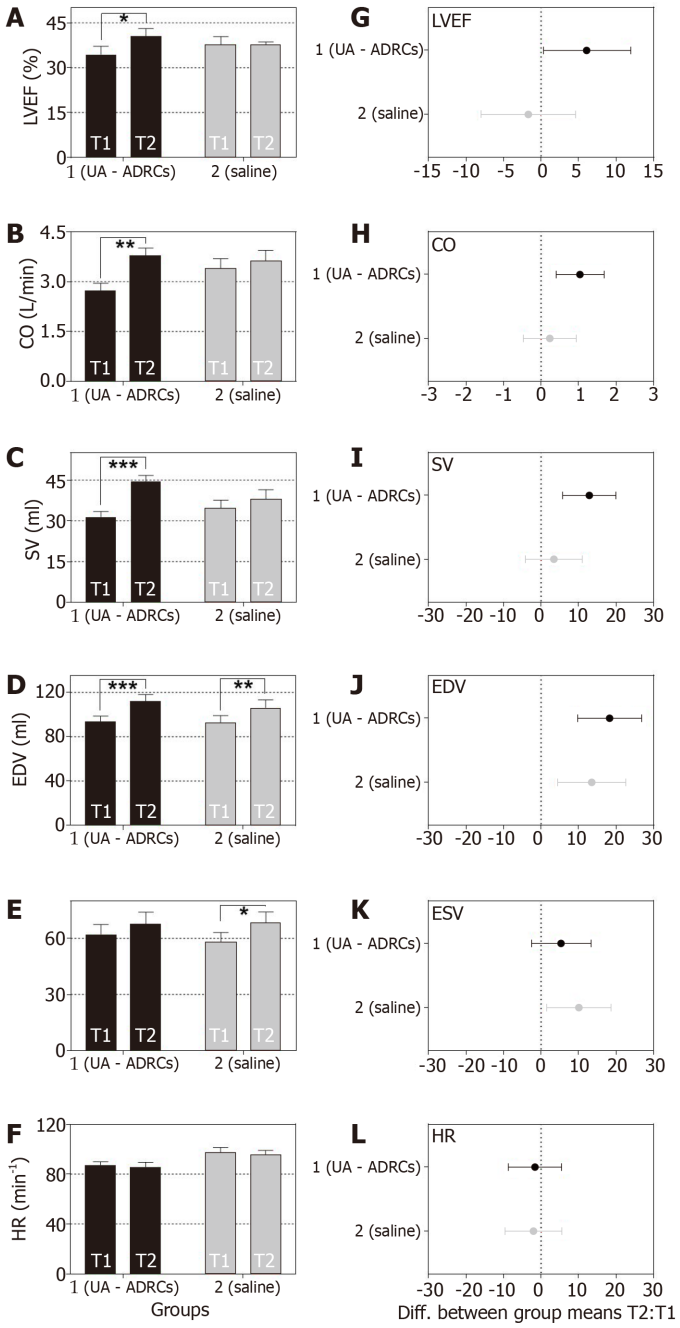

Figure 8 Change in cardiac function after delivery of UA-ADRCs or saline. The panels show group-specific mean ± SE of (A) left ventricular ejection fraction (LVEF, (B) cardiac output (CO), (C) stroke volume (SV), (D) end-diastolic volume (EDV), (E) end-systolic volume (ESV) and (F) heart rate (HR) of animals in group 1 (delivery of UA-ADRCs) (green bars) and group 2 (delivery of saline as control) (red bars) at 4 wk after infarction (T1) and 6 wk later (T2). *P* values of repeated measures two-way analysis of variance are provided in Table 1; results of group-specific Bonferroni's multiple comparison tests are indicated ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$). 95% confidence intervals (Bonferroni) of the differences of group-specific mean data between T2 and T1 are shown in (G-L). UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

delivery of allogeneic BMSCs than after delivery of autologous BMSCs: Different mean age of the donors (the donors in the allogeneic BMSC group were on average approximately half as old as the donors in the autologous BMSC group; mean age of the latter: approximately 57.4 years), possible adverse impact of the disease milieu (including the proinflammatory phenotype), and the possibility of enhanced endogenous cardio-repair after delivery of allogeneic BMSCs in chronic NIDCM. However, specific data supporting these hypotheses were not provided in this study[47]. At least the age of the donors itself may not explain the different clinical outcome in the POSEIDON-DCM RCT[47]. A recent study showed that protein expression profiles of human umbilical vein endothelial cells (HUVECs) that were co-

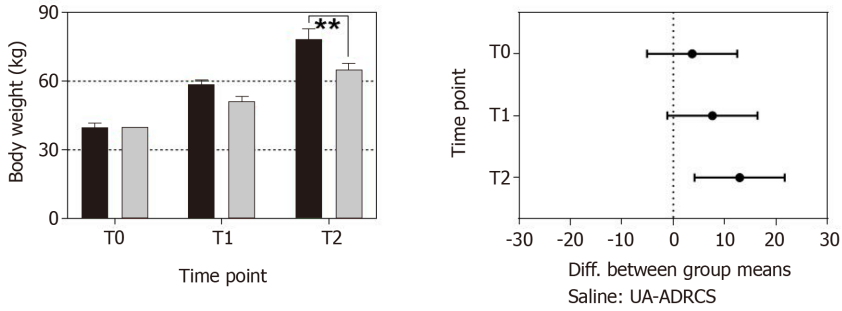

Figure 9 Change in body weight after delivery of UA-ADRCs or saline. The left panel shows group-specific mean ± SE of the body weight of animals in group 1 (delivery of UA-ADRCs) (green bars) and group 2 (delivery of saline as control) (red bars) at baseline (T0), 4 wk after infarction (T1) and 6 wk later (T2). In both groups, the mean body weight significantly increased during the investigated period (group 1: +48% from T0 to T1 and +97% from T0 to T2; group 2: +42% from T0 to T1 and +81% from T0 to T2; $P_{\text{Interaction}} = 0.076$; $P_{\text{Time}} < 0.001$; $P_{\text{Treatment}} = 0.009$; $P_{\text{Subjects (matching)}} = 0.007$). Post hoc Bonferroni tests for pairwise comparisons demonstrated a significant difference in mean body weight between the groups at T2 ($^{b}P < 0.01$) but not at T0 or T1. 95% confidence intervals (Bonferroni) of the differences of group-specific mean data are displayed in the right panel. UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

cultured under oxidative stress conditions with UA-ADRCs from healthy persons aged between 42 and 47 years did not differ from protein expression profiles of HUVECs that were co-cultured under identical conditions with ADRCs from healthy persons aged between 61 and 62 years[48]. Nevertheless, one might speculate that the cardiac repair potential of BMSCs obtained from patients suffering from NIDCM for several years (with a reduction in LVEF by more than 50% compared to healthy people[36,49] could differ significantly from the cardiac repair potential of BMSCs obtained from young, healthy people. In any case, the outcome of the POSEIDON-DCM RCT[47] cannot be directly compared to the outcome of treatment of induced chronic MI in porcine animal models (Figure 14). In fact, a RCT on patients who have suffered from chronic IHD with an averaged time of approximately 11 years since the last MI and mean LVEF of approximately 27% at baseline (POSEIDON)[50] reported almost no improvement of LVEF 13 mo after transendocardial delivery of up to 200 × 10^6 allogeneic BMSCs (averaged +1.7% absolute change compared to baseline) or up to 200 × 10^6 autologous BMSCs (averaged +2.3% absolute change compared to baseline). The disappointing outcome of the autologous BMSC group in the POSEIDON RCT[50] may be due to the considerably prolonged time between the last MI and the delivery of cells (on average 12.8 years; range: 2.4-31.8), which may be clinically less relevant than the time between induced MI and the delivery of UA-ADRCs investigated in the present study (4 wk).

Several cell delivery routes were tested in preclinical studies and clinical trials in order to optimize the efficacy of mesenchymal stem cell therapy for MI[51]. A recent meta-analysis of these studies indicated that transendocardial stem cell injection (TESI) may be superior to intramyocardial (transpericardial) injection, intracoronary infusion or intravenous infusion[51]. However, TESI may preferentially reduce scar size and functional response of the ventricular wall at local stem cell injection sites, as observed in the POSEIDON RCT[52]. Thus, TESI might not be the best cell delivery route.

In order to overcome the natural endothelial barrier, we applied in the present study a novel cell delivery method by applying increased pressure to extravasate the cells (retrograde delivery of cells into the temporarily blocked LAD vein using a standard over-the-wire balloon catheter). Another published study on pigs (occlusion of the LAD artery for 45 min; delivery of 1 × 10^7 ^{111}indium-oxine–labeled autologous ASCs 6 d later; delivery of the cells into a 4 cm long segment of the LAD vein using a double-balloon catheter within on average 8 seconds; named RCV delivery) showed that RCV delivery also resulted in increased amounts of cells retained in the heart at 1 h and 24 h after delivery (mean relative amount of ^{111}indium-oxine retention: 18% after 1 h and 19% after 24 h)[53]. Of note, these data cannot be directly compared to the experimental model investigated in the present study (occlusion of the LAD artery for 180 min; delivery of UA-ARDCs 4 wk later). We hypothesize that delivery of cells into a temporarily blocked vessel – with continuing injection and the resulting increase in pressure in the compartment between the balloons blocked segment of the vessel and the capillaries – enables cells to overcome the endothelial barrier. It might be a task of

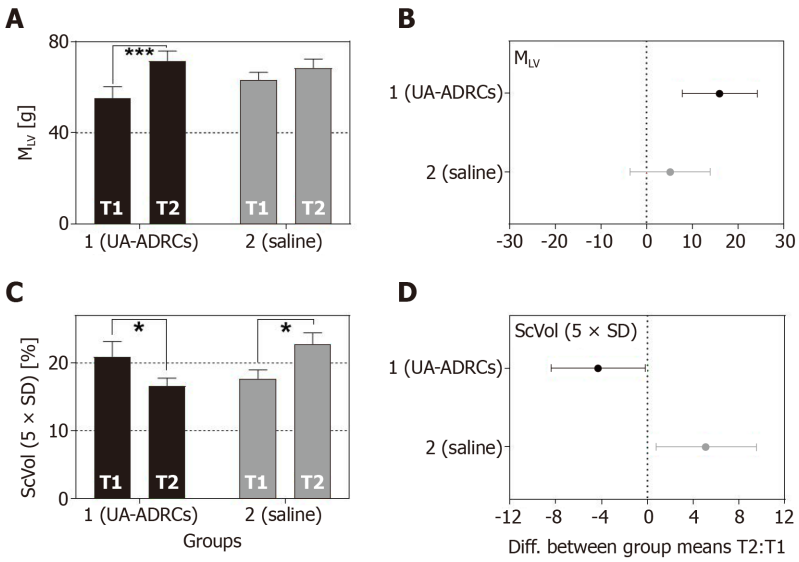

Figure 10 Change in cardiac structure after delivery of UA-ADRCs or saline. The panels show group-specific mean ± SE of (A) the left ventricular mass (M_{LV}) and (B) the relative amount of scar volume of the left ventricular wall (ScVol) of animals in group 1 (delivery of UA-ADRCs) (green bars) and group 2 (delivery of saline as control) (red bars) at 4 wk after infarction (T1) and 6 wk later (T2). *P* values of repeated measures two-way analysis of variance are provided in **Table 1**; results of group-specific Bonferroni's multiple comparison tests are indicated ([a]*P* < 0.05; [c]*P* < 0.001). 95% confidence intervals (Bonferroni) of the differences of group-specific mean data between T2 and T1 are shown in (C, D). UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

following studies to further investigate to which extent the novel delivery mode contributed to improvements in cardiac function and structure after induced CMI, as reported here.

The outcome of cell-based therapy for MI depends on the type of cells delivered, which includes the primary origin, way of initial isolation, and processing. The UA-ADRCs used in the present study were neither cultured nor selected or physically, chemically and/or genetically modified. Rather, they were delivered into the heart about one hour after isolating the unmodified cells from the adipose tissue, within the same interventional procedure of cell harvesting and application (*i.e.*, at "point of care").

A number of enzymatic and non-enzymatic systems for isolating UA-ADRCs have been developed[21,54]. The cell yield reported after different procedures varies considerably[21,55]. It was shown that, in general, enzymatic isolation of UA-ADRCs yields significantly more cells than non-enzymatic, mere mechanical isolation[21,56]. In the present study, cells were isolated from adipose tissue using Matrase™ Reagent (InGeneron), an enzyme blend of collagenase I, collagenase II and a recombinantly produced, proprietary neutral protease.

A recent study compared cell yield, cell viability, number of living cells per ml lipoaspirate, biological characteristics, physiological functions and structural properties of UA-ADRCs that were isolated from adipose tissue with the use of Matrase Reagent (Transpose RT/Matrase isolation) (as done in this study) with the same parameters of UA-ADRCs that were mechanically isolated from adipose tissue without the use of Matrase Reagent, but under otherwise identical processing conditions (Transpose RT/no Matrase isolation)[21]. It turned out that, compared to Transpose RT/no Matrase isolation, Transpose RT/Matrase isolation resulted in the following significantly different ($P < 0.05$), clinically relevant effects: (1) An approximately twelve times higher mean number of viable cells per gram processed lipoaspirate; and (2) An approximately 16 times higher number of CFU-Fs per gram lipoaspirate created by the UA-ADRCs[21]. On the other hand, Transpose RT/Matrase isolated UA-ADRCs and Transpose RT/no Matrase isolated UA-ADRCs showed similar, not significantly different ($P > 0.05$) expression levels of the regenerative cell-associated genes Oct4, Hes1 and Klf4[21]. Furthermore, when stimulated with specific differentiation media both Transpose RT / Matrase isolated UA-ADRCs and Transpose RT/no Matrase isolated UA-ADRCs were independently able to differentiate into adipogenic, osteogenic, hepatogenic and neurogenic lineages (*i.e.*, into cells of all three germ layers)[21]. These data demonstrate that (1) the use of Matrase Reagent in isolating UA-ADRCs from adipose tissue did not change

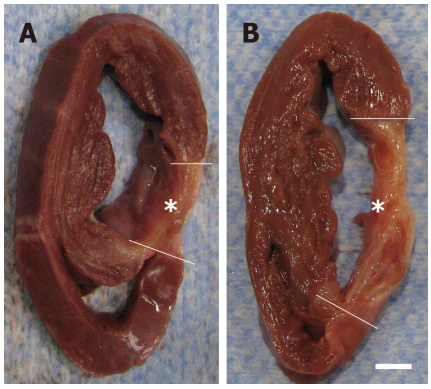

Figure 11 Formation of scar tissue after delivery of UA-ADRCs or saline. A, B: Representative, transversal, 1 cm-thick slices of post mortem hearts from pigs in group 1 (delivery of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells) (A) and group 2 (delivery of saline as control) (B) at T2. The yellow lines indicate the left ventricular border zones of the myocardial infarction (yellow asterisks). The scale bar represents 1 cm. UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

biological characteristics, physiological functions or structural properties of the cells that are of relevance for the intended use (*i.e.*, regeneration, repair or replacement of weakened or injured tissue); (2) the Matrase Reagent did not induce these biological characteristics, physiological functions and structural properties; and (3) the cells were not manipulated by the use of the enzyme, because Transpose RT/Matrase isolated UA-ADRCs and Transpose RT/no Matrase isolated UA-ADRCs showed the same expression of embryonic genes and pluripotent differentiation capacity[21]. These data are highly clinically significant, because safety concerns have recently been raised about the use of cultured adult stem cells in regenerative medicine. In particular, increased rates of potential malignant transformations were observed at higher passages[57-59].

Characterization of cells in this study with flow cytometry yielded the following results. First, 49% of the cells on average expressed CD29 ($CD29^+$), and 37% expressed CD44. Expression of CD29 and CD44 is characteristic of UA-ADRCs[55,60]. With regard to CD29, Mitchell *et al*[61] reported similar results for human UA-ADRCs (mean relative number of $CD29^+$ cells: 48%). In contrast, these authors reported higher mean relative numbers of $CD44^+$ cells than those found in this study (64% *vs* 37%). A potential explanation for this discrepancy may be species-specific differences in the expression of CD44. When culturing UA-ADRCs, Mitchell *et al*[61] observed that the mean relative number of $CD29^+$ cells increased from 71% at passage 1 to 95% at passage 4, and the mean relative number of $CD44^+$ cells increased from 84% at passage 1 to 98% at passage 4. Second, the average number of cells in this study that expressed CD45 (a marker of blood-derived cells[55,62]) was only 9%. Of note, substantially higher mean relative numbers of $CD45^+$ cells were reported for porcine UA-ADRCS (31%[13]) and human UA-ADRCS (30%[61,63]) in the literature. A potential explanation is that no Transpose/Matrase isolation of UA-ADRCs was performed in the latter studies[13,61,63]), further underscoring the superiority of the approach used in this study. Third, the average number of cells in this study that expressed CD31 (a marker of endothelial cells[55,63]) was 11%. A very similar average number of $CD31^+$ cells (8%) was previously reported for porcine UA-ADRCs[13]. In contrast, for human UA-ADRCs, conflicting relative numbers of $CD31^+$ cells were reported in the literature (1%-6% in [63], and 22% on average in[61]). A potential explanation is the use of different isolation procedures in these studies. Fourth, the average number of cells in this study that expressed Oct4 (a transcription factor expressed in human ASCs at passage 1 and is highly associated with stem cell pluripotency[64]) was 4%. Of note, this number could not be compared to results from the other studies discussed here[13,55,60-63] because Oct4 was not investigated in these studies. Fifth, the relative numbers of cells in this study that expressed NG2 and CD146 (which were described in the literature as markers associated with pericytes[55,65]), Nestin (an early marker of neural stem/progenitor cells and proliferative endothelial cells[66]) and CD117 (c-Kit; a marker of common myeloid progenitors, hematopoietic progenitor cells and multipotent progenitors[67]) were 10%, 1%, 4% and 0.3%, respectively. These markers were not investigated in the aforementioned study on UA-ADRCs[13]. Mitchell *et al*[61] only reported an average relative number of 21% of $CD146^+$ cells in human UA-ADRCs. Potential explanations of this discrepancy (1% in this study *vs* 21% in[61]) are species-specific differences, differences in the isolation procedure of UA-ADRCs, or a

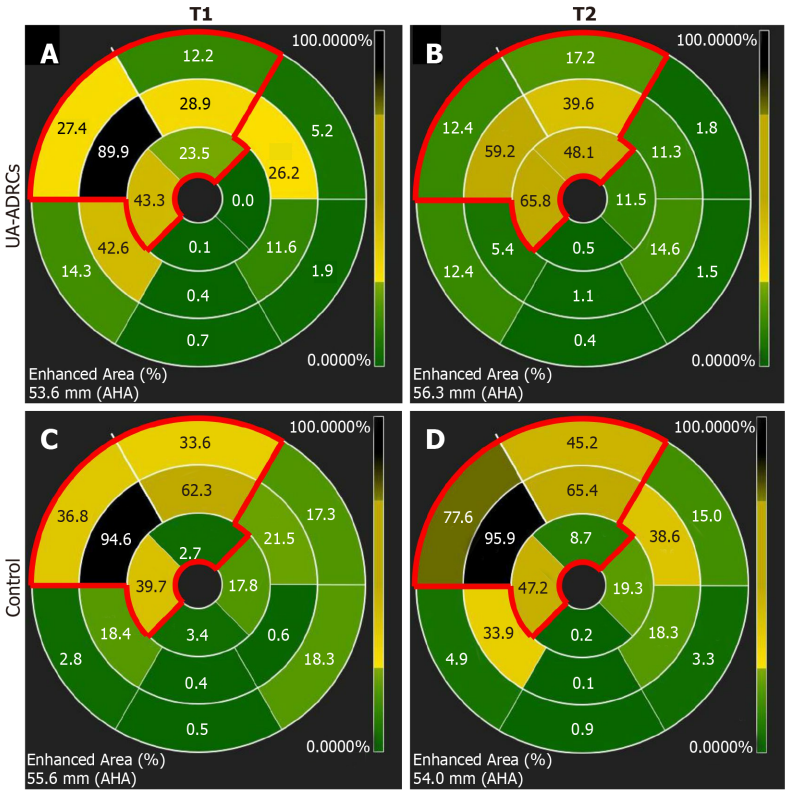

Figure 12 Analysis of regional replacement fibrosis after delivery of UA-ADRCs or saline. The panels visualize data from a representative animal in group 1 (delivery of UA-ADRCs) (A, B) and a representative animal in group 2 (delivery of saline as control) (C, D) at time points T1 (A, C) and T2 (B, D) using color-coded, AHA 17-segment bullseye plots[35]. Segments marked green showed viable myocardium, whereas segments marked black showed complete fibrosis (*i.e.* $NPrel_{SI>5SD}$ > 90%; see main text for details). In all panels, the red lines in the upper left parts of the bullseye plots enclose those segments that are assigned to the territory of the left anterior descending artery in the human heart[35]. The asterisks indicate the mid anteroseptal segment #8 that markedly improved between T1 and T2 after delivery of UA-ADRCs (A, B) but not after delivery of saline as control (C, D). UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

mixture thereof. Collectively, these data support the significance of the UA-ADRCs used in this study to regenerate cardiac tissue in CMI.

It should be mentioned that characterization of the cells delivered in the present study did not follow recommendations published in a joint position statement by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) in 2013 regarding SVF and ASCs[30]. In this joint position statement, it was stated that primary stable positive surface markers for stromal cells would be CD13, CD29, CD34 (> 20%), CD44, CD73 and CD90 (> 40%), whereas primary negative surface markers for stromal cells would be CD31 (< 20%) and CD45 (< 50%)[30]. Furthermore, at least 20% of the SVF would contain a stromal cell population that is immunopositive for the surface marker CD34 and immunonegative for the surface markers CD31, CD45 and CD235a (*i.e.*, $CD31^-/CD34^+/CD45^-/CD235a^-$ cells)[30]. This statement was based on an earlier position statement published by ISCT in 2006 that described "being adherent to plastic, expressing the surface markers CD73, CD90 and CD105, and having the ability to differentiate into osteoblasts, adipocytes and chondrocytes[68]" as the minimal criteria for defining MSCs. However, it should be pointed out that a major shortcoming of this definition of multipotent MSCs is the fact that, for example, fibroblasts also adhere to plastic and express the surface markers CD73, CD90 and CD105, without having the ability to transdifferentiate into other lineages or being MSCs[64]. Furthermore, true pluripotent stem cells do not yet express CD73, CD90 and CD105[24]. Besides this, a recent study compiled the relative amount of ADRCs expressing the surface markers CD13, CD29, CD34, CD44, CD73, CD90, CD31 and CD45, as reported in all studies describing enzymatic and non-enzymatic methods for isolating ADRCs that were published so far[24]. In brief, it was found that (1) the relative amount of $CD34^+$ cells was determined for only very few methods, with substantial variation among methods (ranging between 35%-81%); (2) the relative amount of $CD45^+$ cells varied between 6%-82% among published studies; (3) relative

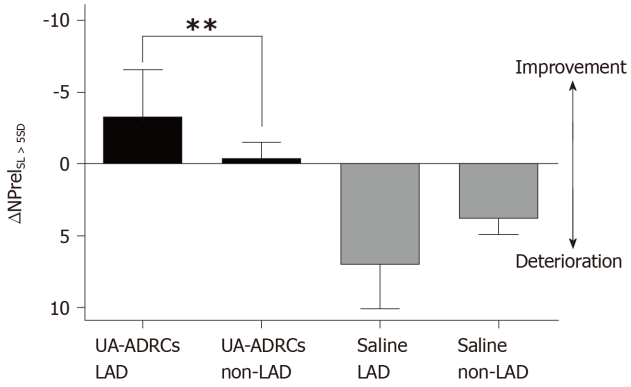

Figure 13 Improvement or deterioration of regional replacement fibrosis after delivery of UA-ADRCs or saline. The panel shows mean and standard error of the mean of the results of replacement fibrosis analysis (as explained in detail in the main text) of segments that are assigned to the territory of the left anterior descending artery in the human heart LAD segments; analysis is also shown for segments that are assigned to the territory of the right coronary artery and the left circumflex coronary artery in the human heart (non-LAD segments) of animals in group 1 [delivery of UA-ADRCs) (green boxes) and group 2 (delivery of saline as control) (red boxes). Data above the zero line indicate improvement, and data below the zero line deterioration. Results of Bonferroni's multiple comparison tests are indicated ($^bP < 0.01$). LAD: Left anterior descending; UA-ADRCs: Fresh, uncultured, unmodified, autologous adipose-derived regenerative cells.

amounts of $CD13^+$ cells, $CD29^+$ cells, $CD44^+$ cells, $CD73^+$ cells, $CD90^+$ cells and $CD31^+$ cells were only determined for a few methods; and (4) the relative amount of $CD31^-/CD34^+/CD45^-/CD235a^-$ cells (as proposed in[30]) was not reported for any method[24]. Collectively, these data render the determination of ADRC surface markers as proposed by IFATS and ISCT[30,68] to not be suitable. This was the reason why no such characterization as proposed by IFATS and ISCT[30,68] was performed in this study.

Unlike cultured cells, UA-ADRCs cannot be labeled. Accordingly, it is more complex to assess the fate of UA-ADRCs after delivery, unlike in the case of allogenic adipose-derived regenerative cells, or in the case of cultured cells that were labeled in culture. Allogenic adipose-derived regenerative cells can be identified in the host tissue using immunohistochemistry[69], and labeled cells can be identified in the host tissue using immunohistochemistry[70], T2* MRI[71] or single-photon emission computed tomography[53]. It is crucial to keep in mind that all this is not possible in the case of UA-ADRCs. It is not possible to collect conditioned medium from UA-ADRCs, nor is it feasible for cultured cells[72]. These factors limit the evaluation of the exact molecular or cellular effects of UA-ADRCs on the therapeutic outcome after MI.

In our study, the delivery of UA-ADRCs in CMI resulted in an increased mean mass of the left ventricle, and a reduced mean relative amount of scar volume of the left ventricular wall (Figures 11-13). In this regard, it is critical to note that in treatments of acute MI, stem cells act *via* different molecular and cellular mechanisms of action than in treatments of chronic MI. In the case of acute MI, the predominant effect of stem cell application may be the prevention of cardiomyocyte apoptosis[73]. However, in our study, delivery of UA-ADRCs into the LAD vein took place 4 wk post-MI. A study on a rat model of MI demonstrated high numbers of active caspase-3 immunopositive (*i.e.*, apoptotic) cells in cardiomyocytes and nonmyocytes at 7-10 d post-MI, but not at 28 d post-MI[8]. Accordingly, in the case of chronic MI, the predominant effect of stem cell application may be the enhancement of the neovascularization response after MI (demonstrated in Figure 5 as well as in[38,40,41]), and the formation of new cardiomyocytes. With regard to the latter, it was a recent key finding that endogenous cardiomyocyte generation can be activated by exercise in the normal and injured adult mouse heart[74]. This obviously does not exclude that the UA-ADRCs applied in this study may also have exerted their function by any (or a combination of several) of the molecular and cellular mechanisms that were described in the literature (such as beneficial effects on the ratio between matrix metalloproteinases and tissue inhibitors of metalloproteinases, reduced collagen expression, inhibited growth of cardiac fibroblasts, and limited local inflammation[75-78]).

Our study has some limitations. One limitation is that only a specific combination of cell type, cell dose, cell delivery route and timing of cell delivery was tested, and this was performed in a pre-determined location of CMI with uniform infarct size. However, it turned out that this specific combination of cell type, dose, delivery route

Table 2 Details of studies that addressed delivery of cultured cells (adipose-derived stem cells, bone marrow-derived stem cells, cardiosphere-derived cells, and c-Kit-positive cardiac stem cells) at a later time after experimentally-induced myocardial infarction in porcine models

Ref.	**Johnston *et al*[38]**
Species	Swine (farm and miniature pigs; age and body weight of the animals not provided)
Duration of LAD occlusion	150 min
Cells	CDCs
Source of cells	
No. of cells	300000 cells/kg body weight
Delivery time	4 wk after MI
Delivery route	Intracoronary
Investigated groups of animals	Group B (group 3 in Figure [14]): 300000 cells/kg body weight
	Group A (group 4 in Figure [14]): Control group
Follow-up	8 wk after delivery of cells (12 wk post-MI)
Use of cardiac MRI	Yes (3T; Siemens, Erlangen, Germany)
mLVEF before MI	
mLVEF after MI	37.8% (group A) and 39.5% (group B)
mLVEF at follow-up	37.6% (group A) and 37.0% (group B)
ΔmLVEF (absolute numbers)	-0.2% (group A) and -1.5% (group B)
ΔmLVEF (relative numbers)	-0.5% (group A) and -6.3% (group B)
mScVol after MI	19.2% (group A) and 17.7% (group B)
mScVol at follow-up	14.2% (group A) and 15.3% (group B)
ΔmScVol (absolute numbers)	-5.0% (group A) and -2.4% (group B)
ΔmScVol (relative numbers)	-26.0% (group A) and -13.6% (group B)
Ref.	**Blázquez *et al*[39]**
Species	Large white pigs (3-4 mo old; body weight 30-35 kg)
Duration of LAD occlusion	90 min
Cells	Allogeneic CDCs
Source of cells	Large White pigs
No. of cells	300000 cells/kg body weight
Delivery time	7 wk after MI
Delivery route	Intrapericardial injection
Investigated groups of animals	Group A (group 5 in Figure [14]): 300000 cells/kg body weight (n = 4)
Follow-up	4 wk after delivery of cells (11 wk post-MI)
Use of cardiac MRI	Yes (1.5 T; Intera, Philips Medical System, Eindhoven, Netherlands)
mLVEF before MI	
mLVEF after MI	39.4
mLVEF at follow-up (4 wk)	40.2
ΔmLVEF (absolute numbers)	+0.8%
ΔmLVEF (relative numbers)	+2.0%
mScVol after MI	10.2%
mScVol at follow-up	8.7%
ΔmScVol (absolute numbers)	-1.5%
ΔmScVol (relative numbers)	-17.2%
Notes	No control group
Ref.	**Tseliou *et al*[40]**
Species	Female Yucatan mini pigs (body weight 40-45 kg); age of the animals not provided
Duration of LAD occlusion	150 min
Cells	Allogeneic CDCs
Source of cells	Male donor Sinclair pigs
No. of cells	12.5×10^6
Delivery time	3 wk after MI
Delivery route	Intracoronary

Investigated groups of animals	Group A: Single-vessel LAD arterial infusion under stop-flow (n = 5)
	Group B: Single-vessel LAD arterial infusion under continuous flow (n = 5)
	Group C: Multi-vessel control group (n = 5)
	Group D: Multi-vessel (LAD, LCX and RCA) under stop-flow (n = 5)
	Group E (group 6 in Figure [14]): Multi-vessel (LAD, LCX and RCA) under continuous flow (n = 6)
	Group F (group 7 in Figure [14]): Control group (n = 5)
Follow-up	7 wk after delivery of cells (4 wk post-MI)
Use of cardiac MRI	Yes (3T; Siemens, Erlangen, Germany)
mLVEF before MI	
mLVEF after MI	47.7% (group A), 43.5% (group B), 46.2% (group C), 48.1% (group D) and 47.4% (group E) and 44.8% (group F)
mLVEF at follow-up	44.9% (group A), 41.6% (group B), 40.3% (group C), 45.6% (group D) and 46.9% (group E) and 37.9% (group F)
ΔmLVEF (absolute numbers)	-2.76% (group A), -1.9% (group B), -5.9% (group C), -2.4% (group D) and -0.5% (group E) and -6.9% (group F)
ΔmLVEF (relative numbers)	-6.1% (group A), -4.5% (group B), -14.5% (group C), -5.3% (group D) and -1.0% (group E) and -18.3% (group F)
mScVol after MI	17.1% (group A), 17.0% (group B), 17.6% (group C), 15.8% (group D) and 14.6% (group E) and 16.0% (group F)
mScVol at follow-up	14.7% (group A), 15.9% (group B), 14.2% (group C), 11.4% (group D) and 11.9% (group E) and 12.1% (group F)
ΔmScVol (absolute numbers)	-2.4% (group A), -1.1% (group B), -3.4% (group C), -4.4% (group D) and -2.7% (group F) and -3.9% (group F)
ΔmScVol (relative numbers)	-16.4% (group A), -6.9% (group B), -24.1% (group C), -38.1% (group D) and -22.5% (group E) and -31.8% (group F)
Ref.	**Dariolli *et al*[41]**
Species	Female *Sus scrofa domestica* pigs (body weight 15-20 kg); age of the animals not provided
Duration of LCX occlusion	Permanent occlusion
Cells	Allogeneic ASCs (passage 4)
Source of cells	Not provided
No. of cells	Between 1×10^6 and 4×10^6
Delivery time	4 wk after MI
Delivery route	Transpericardial intramyocardial injection (20 different sites around the border of MI)
Investigated groups of animals	Group A: 1×10^6 cells/kg body weight (n = 6)
	Group B: 2×10^6 cells/kg body weight (n = 7)
	Group C (group 8 in Figure [14]): 4×10^6 cells/kg body weight (n = 5)
	Group D (group 9 in Figure [14]): Control group (n = 7)
Follow-up	4 wk after delivery of cells (8 wk post-MI)
Use of cardiac MRI	No
mLVEF before MI	
mLVEF after MI	48.4% (group A), 46.7% (group B), 48.7% (group C) and 44.9% (group D)
mLVEF at follow-up	40.6% (group A), 42.2% (group B), 50.0% (group C) and 35.9% (group D)
ΔmLVEF (absolute numbers)	-8.2% (group A), -4.5% (group B), +1.3% (group C) and -9.0% (group D)
ΔmLVEF (relative numbers)	-20.2% (group A), -10.7% (group B), +2.6% (group C) and -25.1% (group D),
mScVol after MI	Not provided
mScVol at follow-up	Not provided
ΔmScVol (absolute numbers)	Not provided
ΔmScVol (relative numbers)	Not provided
Notes	Measurements of LVEF were performed with echocardiography
Ref.	**Natsumeda *et al*[42]**
Species	Female Göttingen swine; age and body weight of the animals not provided
Duration of LAD occlusion	150 min
Cells	Allogeneic BMSCs and allogeneic CSCs
Source of cells	Male Yorkshire swine
No. of cells	Between 1×10^6 and 2×10^8
Delivery time	3 mo after MI

Delivery route	Transendomyocardial intramyocardial injection (TESI) 10 different sites around the border of MI
Investigated groups of animals	Group A: 2×10^8 BMSCs (n = 8)
	Group B: 1×10^6 CSCs (n = 4)
	Group C (group 10 in Figure [14]): 1×10^6 CSCs + 2×10^8 BMSCs (n = 7)
	Group D (group 11 in Figure [14]): Control group (n = 6)
Follow-up	3 mo after delivery of cells (6 mo post-MI)
Use of cardiac MRI	Yes (3T; TIM Trio; Siemens, Erlangen, Germany)
mLVEF before MI	55.3% (group A), 55.2% (group B), 53.8% (group C) and 57.7% (group D)
mLVEF after MI	37.1% (group A), 43.3% (group B), 39.7% (group C) and 41.7% (group D)
mLVEF at follow-up	36.5% (group A), 43.1% (group B), 41.6% (group C) and 40.7% (group D)
ΔmLVEF (absolute numbers)	-0.6% (group A), -0.2% (group B), +1.9% (group C) and -1.0% (group D)
ΔmLVEF (relative numbers)	-1.6%(group A), -0.5% (group B), +4.6% (group C) and -2.5% (group D)
mScVol after MI	16.9% (group A), 12.8% (group B),15.5% (group C) and 17.5% (group D)
mScVol at follow-up	13.7% (group A), 12.4% (group B), 12.5% (group C) and 19.9% (group D)
ΔmScVol (absolute numbers)	-3.2% (group A), -0.4% (group B), -3.0% (group C) and +2.4% (group D)
ΔmScVol (relative numbers)	-23.4% (group A), -3.2% (group B), -24.0% (group C) and +12.1% (group D)

Unlike in the present study, Johnston *et al*[38] identified hyper-enhancement as areas of signal intensity > 2 standard deviations greater than normal myocardium (> 5 standard deviations in the present study). LAD: Left anterior descending artery; LCX: Left circumflex artery; mLVEF: Mean left ventricular ejection fraction; MI: Myocardial infarction; mScVol: Mean relative amount of scar volume of the left ventricular wall.

and timing outperformed the state-of-the-art (Figure 14), highlighting the significance of the results presented in this study. Another limitation is that we did not determine aggregate numbers of cardiomyocytes, which is the only way to unequivocally determine whether a certain therapy increased the number of cardiomyocytes in MI/IHD. The design-based stereologic "optical fractionator" is the state-of-the-art methodology for determining total numbers of cells in an organ[79], and determination of total numbers of cardiomyocytes using the optical fractionator was conclusively demonstrated in the literature[80]. However, to our knowledge, studies on cell-based therapies for MI in which total numbers of cardiomyocytes were investigated using design-based stereology have not yet been published, which is most likely due to the complexity of the necessary histologic analyses. The same applies to the analysis of microvessel density and network complexity as a function of the distance to the myocardial infarction zone, as well as to the determination of proliferating cells (both endogenous and delivered ones) and the quantification of numbers of delivered cells that have differentiated into either cardiomyocytes or cells in vessel walls (the latter requiring analysis of both absolute and relative numbers, related to the total number of cardiomyocytes and the number of cells participating in the formation of vessel walls). Development of adequate methods in quantitative histology/design-based stereology are currently underway, and will allow such studies to be reported in the foreseeable future.

In conclusion, the present study indicates that the delivery of UA-ADRCs by a balloon-blocked retrograde venous injection 4 wk after MI is effective, producing a significant increase in cardiac output and significant reduction in the relative amount of scar volume of the left ventricular wall, without adverse effects occurring during the observation period. Our results could trigger further studies relating to: Evaluation of different doses, delivery route, and timing of UA-ADRCs for treating CMI (including the isolation procedure used) as presented here in future clinical trials under strict criteria, as recently suggested[17].

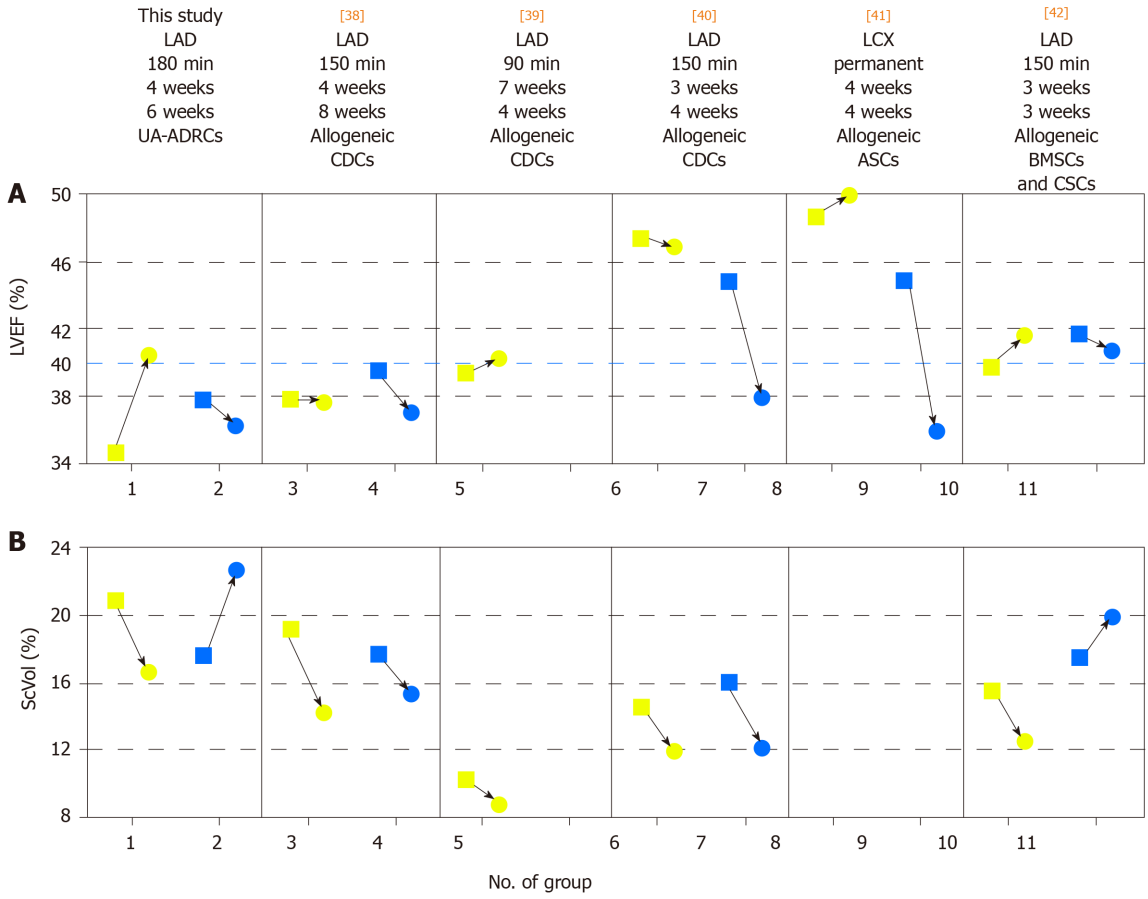

Figure 14 Comparison of studies on cell therapy for chronic MI using porcine models. A, B: Change in mean left ventricular ejection volume (LVEF) (A) and mean relative amount of scar volume of the left ventricular wall (ScVol) (B) from the time immediately before delivery of cells (yellow squares; groups 1, 3, 5, 6, 8 and 10) or control treatment (blue squares; groups 2. 4. 7, 9 and 11), respectively, to follow-up (yellow and blue dots) in the present study (groups 1 and 2) and in all studies on cell therapy for chronic myocardial infarction (> 4 wk) using porcine models that were published to date (groups 3-11; specified in detail in **Table 2**; note that no control group was investigated in[39]). If more than one cell therapy was tested in a study (**Table 2**), the results of the therapy with the most satisfactory outcome are displayed. The information provided on top of Panel A specifies the number of the study in the reference list, the coronary artery that was occluded for experimental MI induction, the duration of occlusion, the interval between experimental MI induction and delivery of cells, and the delivered cell types. The dotted blue line in (A) indicates the border between a moderately reduced LVEF and a reduced LVEF, according to the guidelines for the diagnosis and treatment of acute and chronic heart failure published by the European Society of Cardiology in 2016[36]. MI: Myocardial infarctions; LAD: Left anterior descending artery; LCX: Left circumflex artery; BMSCs: Bone marrow-derived stem cells; CDCs: Cardiosphere-derived stem cells; ASCs: Adipose-derived stem cells; CSCs: Cardiac stem cells; UA-ADRCs: Fresh, uncultured, unmodified autologous adipose-derived regenerative cells.

ARTICLE HIGHLIGHTS

Research background

Cardiovascular diseases substantially contribute to morbidity and mortality worldwide. Myocardial infarction (MI) is one of the most common consequences of ischemic heart disease. Advanced medical treatments and device-based therapies have substantially improved the survival of patients with MI. However, these therapies can only rescue the remaining viable myocardial tissue within the damaged heart, but cannot replace lost myocardium. Accordingly, numerous studies have investigated cell-based therapies for MI. The conflicting results of these studies have established the need for developing innovative approaches for applying cell-based therapy for MI.

Research motivation

Experimental studies on animal models (performed by ourselves and others) demonstrated the potential of fresh, uncultured, unmodified, autologous adipose-derived regenerative cells (UA-ADRCs) for treating acute MI. In contrast, studies on the treatment of chronic MI (CMI; > 4 wk post-MI) with UA-ADRCs have not been published thus far. Our promising results from treating a porcine model with UA-ADRCs for the study of acute MI (*Int J Cardiol* 2010; 144: 26-35) motivated us to investigate the effectiveness and safety of UA-ADRCs for treating CMI in a porcine model. Besides this, as one of several methods for delivering cells to the myocardium, retrograde delivery into a temporarily blocked coronary vein has recently been demonstrated to be an effective option.

Research objectives
Our study aimed to test (in a porcine model for the study of CMI) the following hypotheses: (1) Occlusion of the left anterior descending (LAD) coronary artery for three hours results in a clinically relevant reduction of the left ventricular ejection fraction (LVEF) to less than 40% on an average of 4 wk post-MI (demonstrating significance of the used animal model); (2) Delivery of UA-ADRCs into the LAD vein 4 wk post-MI in this model leads to improved LVEF by more than 15% (relative change) on an average of 10 wk post-MI (primary objective of this study); and (3) The same animal model shows improvements in cardiac structure 6 wk after the delivery of UA-ADRCs (*i.e*. 10 wk post-MI) (secondary objective of this study).

Research methods
The LAD coronary artery of pigs was blocked for 180 min at time point T0. Then, either 18×10^6 UA-ADRCs prepared at "point of care" or saline as control were retrogradely delivered *via* an over-the-wire balloon catheter placed in the temporarily blocked LAD vein 4 wk after T0 (T1). Effects of cells or saline were assessed by cardiac magnetic resonance (CMR) imaging, late gadolinium enhancement CMR imaging and post mortem histologic analysis 10 wk after T0 (T2).

Research results
Unlike the delivery of saline, the delivery of UA-ADRCs demonstrated statistically significant improvements in cardiac function and structure at T2 compared to T1: increased mean LVEF (UA-ADRCs group: +18%; saline group: -4.2%), increased mean cardiac output (UA-ADRCs group: +41%; saline group: +5.9%), increased mean mass of the left ventricle (UA-ADRCs group: +29%; saline group: +8.2%) and reduced mean relative amount of scar volume of the left ventricular wall (UA-ADRCs group: -21%; saline group: +29%).

Research conclusions
The present study indicates that delivery of UA-ADRCs by a balloon-blocked retrograde venous injection 4 wk after MI is effective, producing a significant increase in cardiac output and significant reduction in the relative amount of scar volume of the left ventricular wall, without adverse effects occurring during the observation period.

Research perspectives
Our results justify the evaluation of a new combination of UA-ADRCs (including the isolation procedure), dose, delivery route and timing presented here in future clinical trials for treating CMI under strict criteria, as recently suggested by the European Society of Cardiology Working Group Cellular Biology of the Heart (*Eur Heart J* 2016; 37: 1789-1798), which includes the use of CMR imaging and clinically-relevant endpoints.

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ORIGINAL ARTICLE

Basic Study

Characterization of inflammatory factor-induced changes in mesenchymal stem cell exosomes and sequencing analysis of exosomal microRNAs

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Abstract

BACKGROUND

Treatments utilizing stems cells often require stem cells to be exposed to inflammatory environments, but the effects of such environments are unknown.

AIM

To examine the effects of inflammatory cytokines on the morphology and quantity of mesenchymal stem cell exosomes (MSCs-exo) as well as the differential expression of microRNAs (miRNAs) in the exosomes.

METHODS

MSCs were isolated from human umbilical tissue by enzymatic digestion. Exosomes were then collected after a 48-h incubation period in a serum-free medium with one of the following the inflammatory cytokines: None (control),

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vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor (TNF) α, and interleukin (IL) 6. The morphology and quantity of each group of MSC exosomes were observed and measured. The miRNAs in MSCs-exo were sequenced. We compared the sequenced data with the miRBase and other non-coding databases in order to detect differentially expressed miRNAs and explore their target genes and regulatory mechanisms. *In vitro* tube formation assays and Western blot were performed in endothelial cells which were used to assess the angiogenic potential of MSCs-exo after inflammatory cytokine stimulation.

RESULTS
MSCs-exo were numerous, small, and regularly shaped in the VCAM-1 group. TNFα stimulated MSCs to secrete larger and irregular exosomes. IL6 led to a reduced quantity of MSCs-exo. Compared to the control group, the TNFα and IL6 groups had more downregulated differentially expressed miRNAs, particularly angiogenesis-related miRNAs. The angiogenic potential of MSCs-exo declined after IL6 stimulation.

CONCLUSION
TNFα and IL6 may influence the expression of miRNAs that down-regulate the PI3K-AKT, MAPK, and VEGF signaling pathways; particularly, IL6 significantly down-regulates the PI3K-AKT signaling pathway. Overall, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes.

Key words: Mesenchymal stem cells; Exosomes; MiRNA; Inflammatory cytokines; Angiogenesis

Core tip: The morphology and quantity of mesenchymal stem cell exosomes (MSCs-exo) are impacted in different inflammatory cytokine environments. Inflammatory cytokines impair the ability of MSCs-exo to promote angiogenesis. For instance, the tumor necrosis factor α and interleukin 6 groups exhibited decreased numbers of angiogenesis-related microRNAs (miRNAs), such as miR-196a-5p, miR-17-5p, miR-146b-5p, miR-21-3p, and miR-320. The same groups also had downregulated angiogenesis-related signaling pathways, such as PI3K-AKT and VEGF. Inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes, particularly angiogenesis-related miRNAs.

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INTRODUCTION

Stem cell transplantation has been developing rapidly and has resulted in breakthroughs for the treatment of various diseases. There is especially an interest in the transplantation of mesenchymal stem cells (MSCs), which are tissue-derived cells with self-renewal abilities. Their exosomes (MSCs-exo) not only contain the unique active components of all stem cells, but also are relatively more safe, are more chemically stable, and have the capacity for targeted delivery to biological pathways of interest[1-4].

Exosomes were first found *in vitro* in cultured sheep erythrocyte supernatant[5]. They are membranous vesicles with a diameter of 30-150 nm and a density of 1.10-1.18 g/mL. They are able to affect gene regulation by carrying and releasing various bioactive molecules such as microRNAs (miRNAs) and proteins, both of which can then function as paracrine signaling mediators impacting biological pathways

relevant to disease processes[6]. MiRNAs are non-coding RNAs 22-25 nucleotides in length[7-10]. Exosomes are especially important in producing miRNAs that impact angiogenesis[11].

There is promising research on using MSCs-exo to encourage wound healing and to treat inflammatory arthritis and ischemic diseases. However, in treating these diseases, MSCs are exposed to microenvironments filled with numerous inflammatory cytokines, such as vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor (TNF) α, and interleukin (IL) 6. The effects of these inflammatory cytokines on the morphology and quantity of MSCs-exo and how these effects impact the production of miRNAs and downstream regulatory mechanisms are largely unknown. In this study, we analyzed the effects of VCAM-1, TNFα, and IL6 on the morphology and quantity of MSCs-exo, how these effects enhance differential expression of miRNAs, and how the target genes of these miRNAs and their associated regulatory mechanisms are regulated.

MATERIALS AND METHODS

Cell culture

Human umbilical mesenchymal stem cells were obtained from the Polywin Corporation (Guangzhou, China). The phenotypes of the MSCs were characterized by flow cytometric analysis of cell surface antigens, including tests for the cluster of differentiation (CD)29, CD34, CD44, CD73, and CD105. The MSCs were divided into four groups: Control group: MSCs (1.0×10^5 cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich, United States) medium for 48 h; VCAM-1 group: MSCs (1.0×10^5 cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and VCAM-1 reagent (ADP5, R&D Systems, United States) was added to the medium at a concentration of 20 ng/mL for 48 h; TNFα group: MSCs (1.0×10^5 cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and TNFα reagent (T6674, Sigma-Aldrich) was added to the medium at a concentration of 20 ng/mL for 48 h; and IL6 group: MSCs (1.0×10^5 cells/mL) were cultured in a serum-free DMEM/F12 (Sigma-Aldrich) medium, and IL6 reagent (200-06-20, PeproTech, United States) was added to the medium at a concentration 20 ng/mL for 48 h. The number, distribution, and morphology of cells in each group were observed under a microscope at 100× or 200× magnification for 48 h.

Exosome isolation

Exosomes were isolated from the culture supernatant by ultracentrifugation according to methods described previously[12,13]. Briefly, the culture medium of each group was collected and centrifuged at 2000 × *g* for 10 min at 4 °C. The supernatant was then centrifuged at 10000 × *g* for 10 min at 4 °C. Next, the supernatant was passed through a 0.2-μm filter (Steradisc; Kurabo, Bio-Medical Department, Tokyo, Japan). The filtrate was ultracentrifuged at 100000 × *g* for 70 min at 4 °C (Type 70Ti ultracentrifuge; Beckman Coulter, Inc., Brea, CA, United States). The precipitate was next rinsed with phosphate buffered saline (PBS) and ultracentrifuged at 100000 × *g* for 70 min at 4 °C. The exosome-enriched fraction was next reconstituted in PBS for further studies.

Characteristics and distributions of exosomes in each group were observed. The particle size and concentration of exosomes in each group were measured by nanoparticle tracking analysis.

Differential miRNA analysis and target gene and regulatory signal pathway prediction

The miRNAs of MSCs-exo were sequenced by BGISEQ-500 technology in each group. Sequenced data were compared with miRBase and other non-coding databases. Bioinformatics analysis pipeline steps for miRNA sequencing were: (1) Filtering small RNAs: 18-30 nt RNA segments were separated by polyacrylamide gel electrophoresis (PAGE); (2) 3′ adaptor ligation: A 5-adenylated, 3-blocked single-stranded DNA adapter was linked to the 3′ end of selected small RNAs from step 1; (3) Reverse primer annealing: the RT primer was added to the solution from step 2 and cross-linked to the 3′ adapter of the RNAs and to excess free 3′ adapter; (4) 5′ adaptor ligation: a 5′ adaptor was linked to the 5' end of the product from step 3. The adaptor was attached to the end only, not to the 3′ adaptor or RT primer hybrid chain, thus greatly reducing self-ligation; (5) cDNA synthesis: The RT primers in step 3 were reverse extended to synthesize cDNA strands; (6) PCR amplification: High-fidelity polymerase was used to amplify cDNA, and cDNA with both 3′ and 5′ adaptors was enriched; (7) Library fragment selection: The PCR products of 100-120 bp were separated by PAGE to eliminate primer dimers and other byproducts; (8) Library

quantitative and pooling cyclization; (9) Eliminating the low-quality reads, adaptors and other contaminants to obtain clean reads; (10) Summarizing the length distribution of the clean tags, common, and specific sequences between samples; (11) Assigning the clean tags to different categories; (12) Predicting novel miRNAs; (13) Function annotation of known miRNAs; and (14) Comparing clean reads to the reference base group and other small RNA databases using AASRA software [14], except that Rfam was compared with cmsearch[15]. We used TPM[16] to standardize miRNA expression levels and predicted target genes using RNAhybrid[17], miRanda[18], and TargetScan[19].

Hierarchical clustering analysis showed differentially expressed miRNAs by functional pheatmap. The *P*-values obtained from the differential gene expression tests were corrected by controlling the false discovery rate (FDR)[20] as more stringent criteria with smaller FDRs and bigger fold-change values can be used to identify differentially expressed miRNAs.

Gene ontology (GO) enrichment analysis was performed to identify all GO terms that are significantly enriched in a list of target genes of differentially expressed miRNAs, as well as the genes that correspond to specific biological functions.

The hypergeometric test was then used to find significantly enriched GO terms based on this database (http://www.geneontology.org/). Pathway-based analyses were used to discover the biological functions of target genes using KEGG[21] (the major public pathway-related database).

In vitro Matrigel tube formation assay

HUVECs (4.0×10^4, serum-starved overnight) were seeded in a 96-well plate, cultured in 5% CO_2 overnight, and then treated with PBS (control), control scrambled MSCs-exo, MSCs-exo$^{TNF\alpha}$ (5×10^9, stimulated with TNFα), or MSCs-exo^{IL6} (5×10^9, stimulated with IL6). The plates were previously coated with 150 μL of growth factor-reduced Matrigel (356234, Corning, United States) in serum-free medium. Tube formation ability of control or MSCs-exo-treated HUVECs was examined by determining the total number of tubes formed and branching points in 4 to 6 h. Each condition in each experiment was assessed at least in duplicate.

Western blot analysis

HUVECs (4.0×10^4) were treated with PBS (control), control scrambled MSCs-exo, MSCs-exo$^{TNF\alpha}$ (5×10^9, stimulated with TNFα), or MSCs-exo^{IL6} (5×10^9, stimulated with IL6). The effect of MSCs-exo treatment on PI3K-AKT and MAPK, which are related to angiogenic signaling, was examined by measuring the expression of AKT (1:1000 dilution, 4691S, Cell Signaling Technology, Danvers, MA, United States), phospho-AKT (1:1000 dilution, 13038S, Cell Signaling Technology), phospho-p44/42 MAPK (Erk1/2) (1:2000 dilution, 4370S, Cell Signaling Technology), and p44/42 MAPK (Erk1/2) (1:1000 dilution, 4695S, Cell Signaling Technology) in endothelial cells by Western blot. Each condition in each experiment was assessed at least in triplicate.

Statistical analysis

The data from each group were collected and analyzed using SPSS 11.5 software (IBM SPSS China, Shanghai, china). Numerical data are presented as the mean ± SE; comparisons between groups were evaluated by Student's *t*-test or ANOVA, with $P < 0.05$ considered significant.

RESULTS

Phenotypic characterization of MSCs

Cell purity (85% to 95%) was determined *via* flow cytometry. The cells were positive for mesenchymal cell markers such as CD29, CD44, CD73, and CD105 and negative for hematopoietic cell markers such as CD34 and HLA-DR (Figure 1).

Effect of inflammatory cytokines on MSCs-exo

The VCAM-1 group had small regularly shaped MSCs-exo. The TNFα group had large irregularly shaped MSCs-exo. The IL6 group had medium regularly shaped MSCs-exo (Figure 2)

The density of MSCs-exo was 7.42×10^8/mL in the control group, 1.10×10^9/mL in the VCAM-1 group, 7.37×10^8/mL in the TNFα group, and 3.01×10^8/mL in the IL6 group (Figure 3).

Effect of differential miRNA expression, secondary to inflammatory cytokine exposure, on biological function

Correlation analyses showed that the miRNA expression profiles in the IL6 group

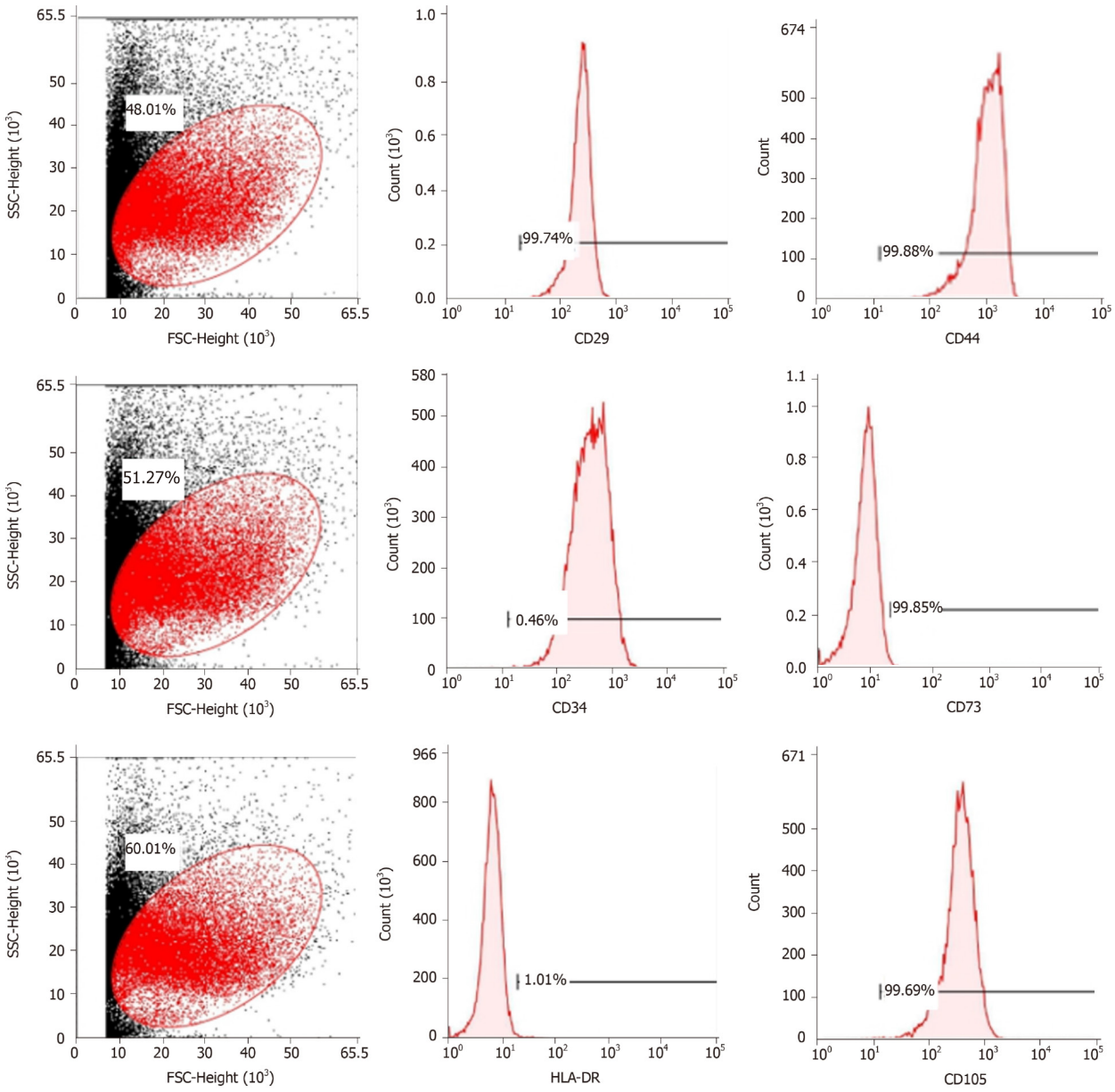


Figure 1 Flow cytometric analysis of mesenchymal stem cell-related cell surface markers. High expression of positive mesenchymal cell markers (CD29, CD44, CD73, and CD105), and low expression of negative cell markers such as CD34 and HLA-DR were observed. n = 3 to 5; P < 0.05,

(0.583) and TNFα group (0.697) were more different from that of the control group than that in the VCAM1 group (0.985) (Figures 4 and 5). The top 10 miRNAs in each group are shown in Table 1. Many miRNAs, particularly some important angiogenesis-related miRNAs, were downregulated in the TNFα group and IL6 group compared to the control group (Tables 2-4 and Figure 6). Hierarchical clustering indicated that the expression levels of the majority of miRNAs in the IL6 group were downregulated compared with those of the control group (Figure 7). According to GO enrichment analysis, miRNAs in exosomes exposed to inflammatory cytokines, compared to controls, had a different regulatory effect on cellular components, molecular function, and biological processes (Figure 8). More specifically, pathway enrichment analysis showed that the target genes of the differentially expressed miRNAs, including those related to angiogenesis, differed among the four groups (Figures 9 and 10). The following angiogenesis-related pathways were more down-regulated in the TNFα and IL6 groups than in the control group: The PI3K-AKT signaling pathway (Q = 0.0978197212 and 0.0581120875 in the TNFα group and IL6 group, respectively), the MAPK signaling pathway (Q = 0.5775485 and 0.9837761532 in the TNFα group and IL6 group, respectively), and the VEGF signaling pathway (Q = 0.4082212190 and 0.1711566 in the TNFα group and IL6 group, respectively) (Figures 10-13).

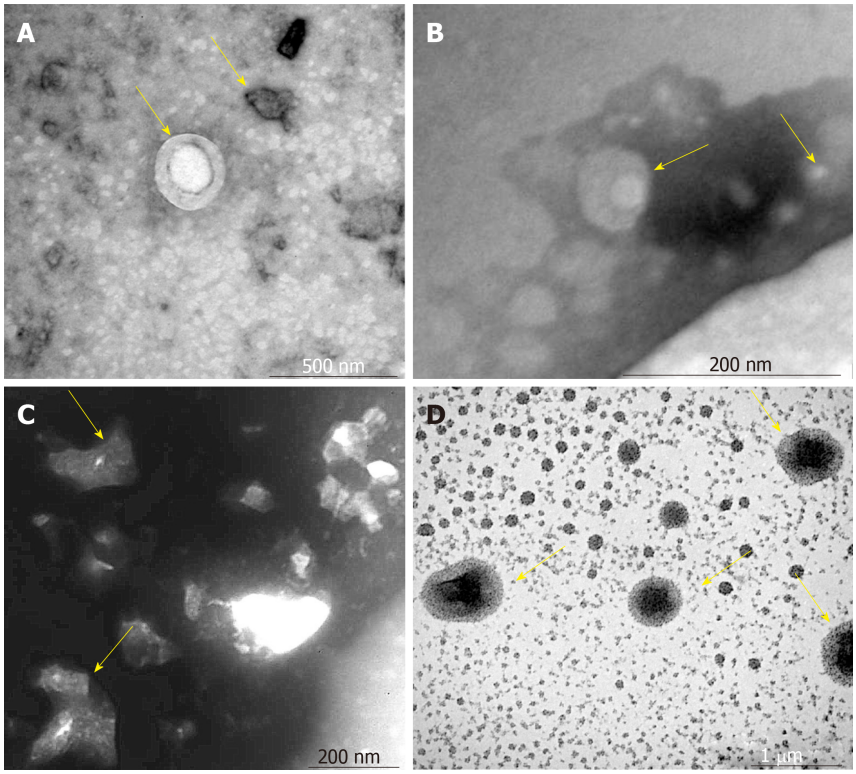


Figure 2 Morphology of mesenchymal stem cell exosomes (magnification, ×65000). A-D: Mesenchymal stem cell exosomes (MSCs-exo) in (A) the control group (yellow arrow), (B) the vascular cell adhesion molecule-1 group (yellow arrow), (C) the tumor necrosis factor α group (yellow arrow), and (D) the interleukin 6 group (yellow arrow).

MSCs-exo promote endothelial cell angiogenesis

The ability of MSCs-exo to enhance tube formation was assessed using a Matrigel assay. MSCs-exo, on average, caused an increase in tube formation and branching ($P < 0.05$; Figure 14) compared to the untreated control group. The angiogenesis effect of MSCs-exo stimulated with TNFα and IL6 was lower than that cultured without TNFα or IL6. This finding confirmed that MSCs-exo can promote angiogenesis (Figure 14).

MSCs-exo stimulated with IL6 inhibit PI3K-AKT signaling pathway activation in endothelial cells

We observed a decline in the expression of phosphorylated AKT (pAKT) in endothelial cells after MSCs-exo were stimulated with IL6, although MSC-EV treatment had no effect on the expression of their unphosphorylated forms (Figure 15).

DISCUSSION

MSCs have been used to treat cardiovascular diseases (CVDs) and represent a promising cell-based therapy for regenerative medicine and the treatment of inflammatory and autoimmune diseases[22]. Their treatment efficacy hinges on their ability to alter disease-specific pathways *via* secreted miRNAs, so it is imperative to understand how disease environments, which often are inflammatory, can impact secreted miRNAs and thus potentially their treatment efficacy. MSCs-exosomes are used for treating CVDs such as acute myocardial infarction, stroke, pulmonary hypertension, and septic cardiomyopathy[23]. Biological properties of MSCs-exo have rendered them as a new strategy for wound regeneration and ischemic disease[24-26]. MSCs-exo exert an anti-inflammatory effect on T and B lymphocytes independently of MSCs priming. The potential therapeutic effects also were demonstrated in inflammatory arthritis[27].

Ischemic diseases, trauma, and immunological diseases are all accompanied by inflammatory reactions, and a large number of inflammatory cytokines including VCAM-1, TNFα, and IL6 are involved in the progression of these diseases. T cells are activated dependent on VCAM-1 interactions[28]. VCAM-1 plays a "backup" role in hASC contact-dependent immune suppression[29]. TNFα is a multifunctional cytokine

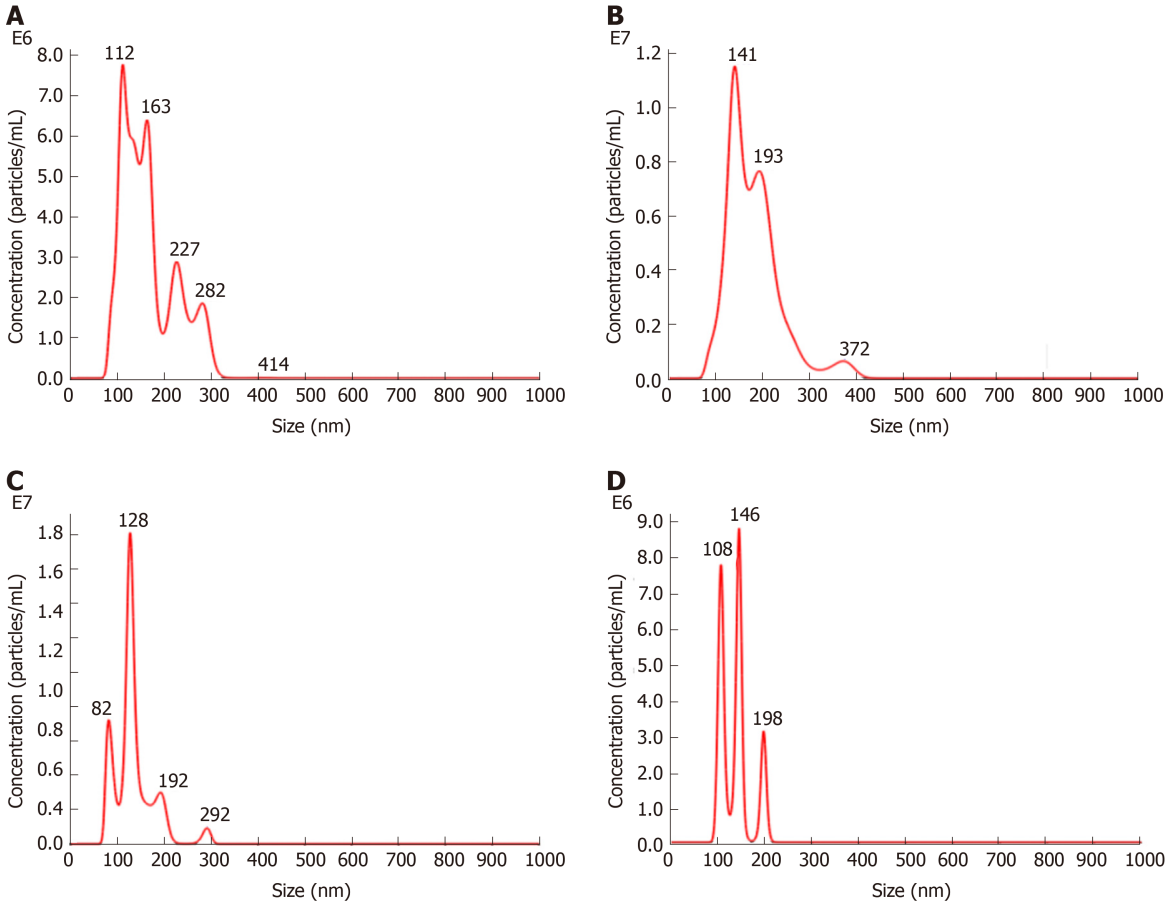


Figure 3 Nanoparticle tracking analysis. A-D: Density of exosomes of different sizes in (A) the control group, (B) the vascular cell adhesion molecule-1 group, (C) the tumor necrosis factor α group, and (D) the interleukin 6 group.

that acts as a central biological mediator for critical immune functions, including inflammation, infection, and antitumor responses[30]. IL6 plays an important role in the inflammatory response following hypoxic-ischemic encephalopathy[31]. Therefore, the biological properties of MSCs-exos and their therapeutic effects need to be studied together with the inflammatory factors. A large amount of evidence suggests that the effect of MSCs-exo therapeutics will be affected by the inflammatory environment with regard to most of CVDs and ischemic diseases[32-40].

Some previous reports showed[41] the effect of stimulation with cytokines interferon γ and TNFα on adipose MSCs (AMSCs). Pro-inflammatory stimuli could enhance the immunosuppressive functions of AMSC-derived exosomes. There was an increase in the expression of miRNAs (miR-34a-5p, miR21, and miR146a-5p) in exosomes produced by pre-activated AMSCs compared to those released by untreated cells.

The present study also found that the inflammatory cytokines VCAM-1, TNFα, and IL6 impact the size and morphology of MSCs-exo as well as the diversity of miRNAs they can produce, especially miRNAs impacting angiogenesis. According to GO enrichment analysis, miRNAs in exosomes exposed to inflammatory cytokines, compared to controls, had a different regulatory effect on cellular proliferation and differentiation, molecular signal transduction, immunosuppressive functions, angiogenesis and so on.

Some observed effects suggested that inflammatory cytokines impaired the ability of MSCs-exo to promote angiogenesis. For example, the TNFα and IL6 groups exhibited decreased numbers of angiogenesis-related miRNAs, such as miR-196a-5p, miR-17-5p, miR-146b-5p, miR-21-3p, and miR-320. The same groups also had downregulated angiogenesis-related signaling pathways, such as PI3K-AKT, MAPK, and VEGF. However, other effects suggested that inflammatory cytokines may promote the ability of MSCs-exo to encourage angiogenesis. Exosomes contained hsa-mir-4488, hsa-mir-671-5p, and hsa-mir-4446-3p after VCAM-1 stimulation, hsa-mir-4488, hsa-mir-671-5p, and hsa-miR-497-5p after TNFα stimulation, and hsa-mir-4488, hsa-miR-145-5p, and hsa-miR-1260a after IL6 stimulation, all of which promote angiogenesis. More specifically, hsa-miR-671-5p encourages NM_006500.2 to produce the downstream product VEGFb, which activates the VEGF pathway and thereby

Table 1 Differentially expressed miRNAs

Top 10 differentially expressed miRNAs										
Control group			VCAM-1 group			In the TNFα group			In the IL6 group	
sRNA id	Count (16379497)	TPM	sRNA id	Count (23095609)	TPM	sRNA id	Count (20739574)	TPM	sRNA id	Count (32116232)
miR-21-5p	1544110	76933.99	miR-21-5p	1640334	70885.38	miR-21-5p	1454726	70066.87	miR-21-5p	1323387
miR-127-3p	159283	9724.54	miR-127-3p	259830	11250.19	miR-100-5p	408192	19681.79	miR-199a-3p,miR-199b-3p	317377
miR-199a-3p,miR-199b-3p	139220	8499.65	miR-199a-3p, miR-199b-3p	155376	6727.51	miR-127-3p	321306	15492.41	miR-127-3p	300549
miR-100-5p	132865	8111.67	miR-100-5p	140774	6095.27	miR-199a-3p, miR-199b-3p	271902	13110.3	miR-100-5p	265054
miR-222-3p	89564	5468.06	miR-26a-5p	107800	4667.55	miR-23a-3p	140623	6780.42	miR-34a-5p	178542
miR-26a-5p	77524	4732.99	miR-34a-5p	86786	3757.68	miR-222-3p	129932	6264.93	miR-23a-3p	144385
miR-34a-5p	76681	4681.52	miR-451a	77297	3346.83	miR-146a-5p	128078	6175.54	miR-222-3p	111957
miR-181a-5p	67287	4108	miR-23a-3p	71930	3114.44	miR-34a-5p	119952	5783.73	miR-146a-5p	100038
miR-411-5p	56606	3455.91	miR-196a-5p	63939	2768.45	miR-143-3p	101398	4889.11	miR-26a-5p	98114
miR-143-3p	49739	3036.66	miR-181a-5p	62752	2717.05	miR-26a-5p	99628	4803.76	miR-181a-5p	97685

Count is the number of mapped tags, and the value in parentheses is the total number. Transcripts per kilobase million is the standardized expression value. VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6; TPM: Transcripts per kilobase million.

promotes angiogenesis. Hsa-miR-671-5p also encourages NM_001773.2 to produce CD34, which in turn activates the PI3K-AKT pathway, this promoting cell proliferation and angiogenesis. It is unclear what the ultimate results of these competing effects on angiogenesis would be.

Our study demonstrated that MSCs-exo perhaps induced HUVECs to form capillary-like structures *in vitro*. The effect of MSCs-exo in promoting angiogenesis would be reduced when the stem cells were subjected to TNFα and IL6 stimulation. Besides endothelial cell angiogenesis-related molecular expression, functional characteristics such as the PI3K-AKT signaling pathway may be down-regulated in MSCs-exo that were stimulated with IL6.

The major limitation of this study is that it was conducted *in vitro*. Further research needs to be conducted *in vivo* using an animal model that more closely mirrors the inflammatory environment to which MSCs are exposed when administered for treatment in humans.

In conclusion, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological processes. Further *in vivo* research needs to be conducted to explore how the treatment efficacy of MSCs is impacted by these inflammatory-induced changes in exosomes and their miRNAs.

Table 2 Top 10 differentially expressed miRNAs in each group (Poisson distribution screening)

Comparison of top 10 differentially expressed miRNAs																										
VCAM-1 group *vs* control group									TNFα group *vs* control group								IL6 group *vs* control group									
miRNA id	Count (control)	Count (VCAM-1)	TPM (control)	TPM (VCAM-1)	Log2 ratio (VCAM-1/control)	Up/down-regulation (VCAM-1/control)	*P*	FDR	miRNA id	Count (control)	Count (TNFα)	TPM (control)	TPM (TNFα)	Log2 ratio (TNFα/control)	Up/down-regulation (TNFα/control)	*P*	FDR	miRNA id	Count (control)	Count (IL6)	TPM (control)	TPM (IL6)	Log2 ratio (IL6/control)	Up/down-regulation (IL6/control)	*P* value	FDR
miR-146a-5p	20510	5173	1252.18	223.98	-2.483	Down	0	0	miR-146a-5p	20510	128078	1252.18	6175.54	2.302123	Up	0	0	miR-3529-3p	473	8930	28.88	278.05	3.267202	Up	0	0
miR-149-5p	4558	2391	278.27	103.53	-1.42644	Down	0	0	miR-342-3p	2117	7130	129.25	343.79	1.411363	Up	0	0	miR-320c	1276	7983	77.9	248.57	1.673957	Up	0	0
miR-222-3p	89564	48444	5468.06	2097.54	-1.38233	Down	0	0	miR-23a-3p	42777	140623	2611.62	6780.42	1.37643	Up	0	0	miR-24-3p	11151	50968	680.79	1586.99	1.221011	Up	0	0
miR-29a-3p	8451	5054	515.95	218.83	-1.23742	Down	0	0	miR-100-5p	132865	408192	8111.67	19681.79	1.278791	Up	0	0	miR-7-5p	16653	101	1016.7	3.14	-8.33891	Down	0	0
miR-451a	2760	77297	168.5	3346.83	4.311975	Up	0	0	miR-337-3p	5655	16027	345.25	772.77	1.162398	Up	0	0	miR-196a-5p	26485	11440	1616.96	356.21	-2.18248	Down	0	0
miR-490-5p	1839	475	112.27	20.57	-2.44836	Down	2.00E-305	1.20E-303	miR-409-3p	4207	11169	256.85	538.54	1.068127	Up	0	0	miR-451a	2760	1658	168.5	51.62	-1.70675	Down	0	0
miR-1246	1812	6191	110.63	268.06	1.276813	Up	1.39E-276	8.02E-275	miR-221-3p	4067	10541	248.3	508.26	1.033482	Up	0	0	miR-379-5p	13430	8083	819.93	251.68	-1.70391	Down	0	0
miR-181a-2-3p	3400	2008	207.58	86.94	-1.25558	Down	2.31E-219	1.18E-217	miR-7-5p	16653	136	1016.7	6.56	-7.27598	Down	0	0	miR-196b-5p	18735	15676	1143.81	488.1	-1.2286	Down	0	0
miR-204-5p	3116	1757	190.24	76.08	-1.32223	Down	3.36E-218	1.66E-216	miR-196a-5p	26485	8359	1616.96	403.05	-2.00425	Down	0	0	miR-424-5p	12774	11917	779.88	371.06	-1.0716	Down	0	0
miR-378e	2	817	0.12	35.37	8.203348	Up	4.36E-186	2.00E-184	miR-26b-5p	6496	3410	396.59	164.42	-1.27026	Down	0	0	miR-26b-5p	6496	6086	396.59	189.5	-1.06545	Down	0	0

P-values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

Table 3 Comparison of differentially expressed angiogenesis-related miRNAs between the TNFα group and control group

miRNA id	Count (control)	Count(TNFα)	TPM (control)	TPM (TNFα)	Log2 ratio (TNFα/ control)	Up/ down-regulation (TNFα/ control)	*P*	FDR
hsa-miR-196a-5p	26485	8359	1616.96	403.05	-2.004253263	Down	0	0
hsa-miR-320e	161	46	9.83	2.22	-2.14663174	Down	5.95E-23	6.85E-22

P-values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; TNF: Tumor necrosis factor.

Table 4 Comparison of differentially expressed angiogenesis-related miRNAs between the IL6 group and control group

miRNA id	Count (control)	Count (IL6)	TPM (control)	TPM (IL6)	Log2 ratio (IL6/control)	Up/down-regulation (IL6/control)	*P*	FDR
hsa-miR-196a-5p	26485	11440	1616.96	356.21	-2.182484067	Down	0	0
hsa-miR-17-5p	252	138	15.39	4.3	-1.839584667	Down	2.24E-35	2.98E-34
hsa-miR-146b-5p	78	44	4.76	1.37	-1.79678568	Down	1.08E-11	7.53E-11
hsa-miR-21-3p	440	358	26.86	11.15	-1.268415595	Down	4.66E-35	6.02E-34
hsa-miR-320e	161	153	9.83	4.76	-1.046229843	Down	1.78E-10	1.11E-09

P-values were corrected. TPM: Transcripts per kilobase million; FDR: False discovery rate; IL6: Interleukin 6.

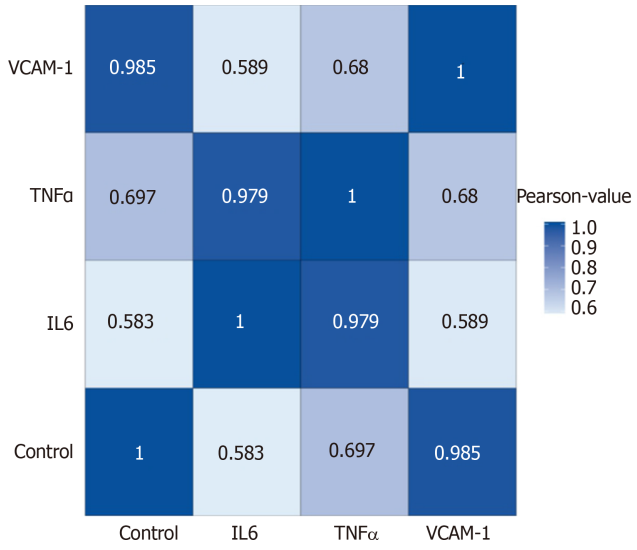


Figure 4 Correlation analyses between groups. Blue color represents the correlation coefficient (the deeper the blue, the stronger the correlation). VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

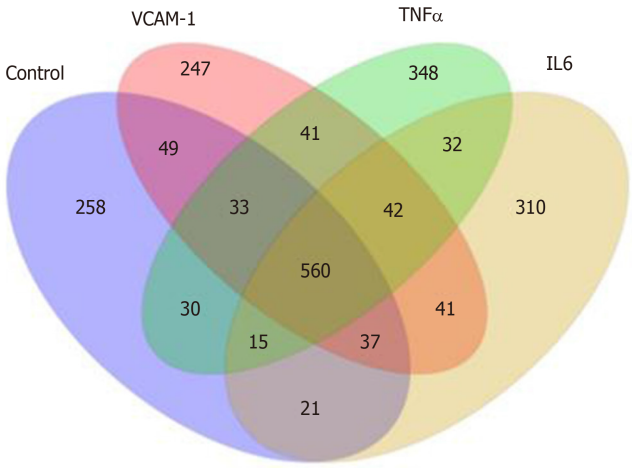


Figure 5 Distribution of differentially expressed miRNAs in different groups. VCAM-1: Vascular cell adhesion molecule-1; TNF: Tumor necrosis factor; IL6: Interleukin 6.

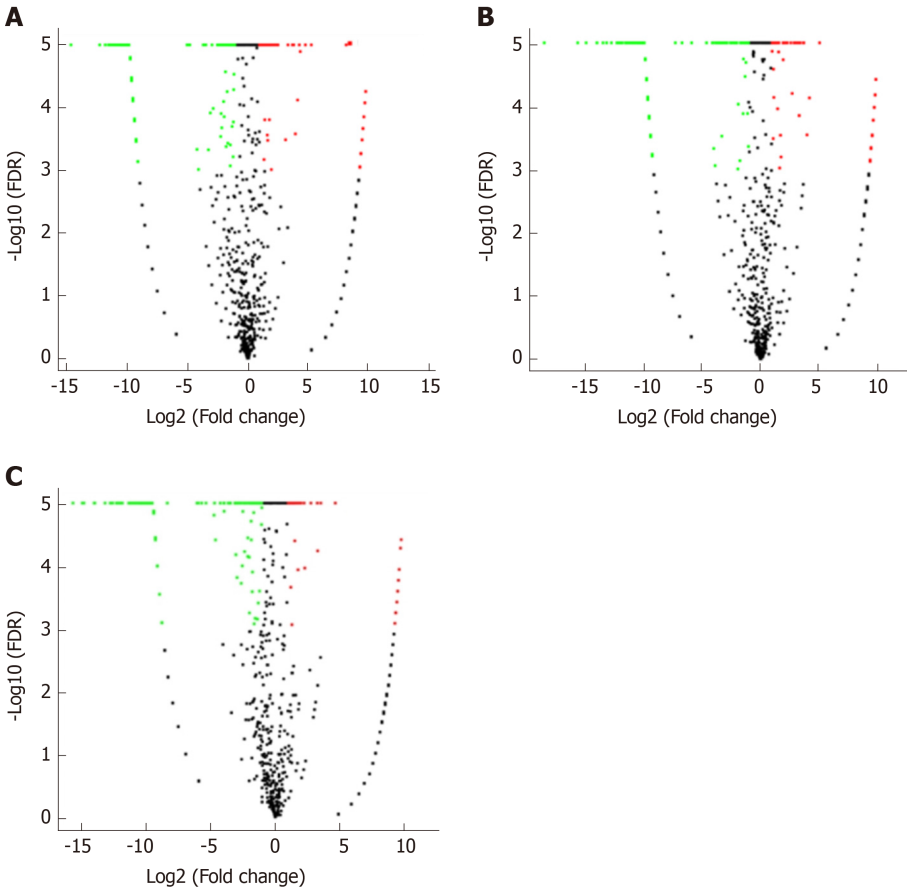


Figure 6 Volcano plots. A-C: Volcano plots of (A) vascular cell adhesion molecule-1 group *vs* control group, (B) tumor necrosis factor α group *vs* control group, and (C) interleukin 6 group *vs* control group. The X axis is the log2(Fold change), and the Y axis is the -log10(FDR), with green points indicating down-regulation [log2(Fold old change) ≤ -1 and FDR ≤ 0.001] and red points indicating upregulation [log2(Fold change) ≥ 1 and FDR ≤ 0.001]. FDR: False discovery rate.

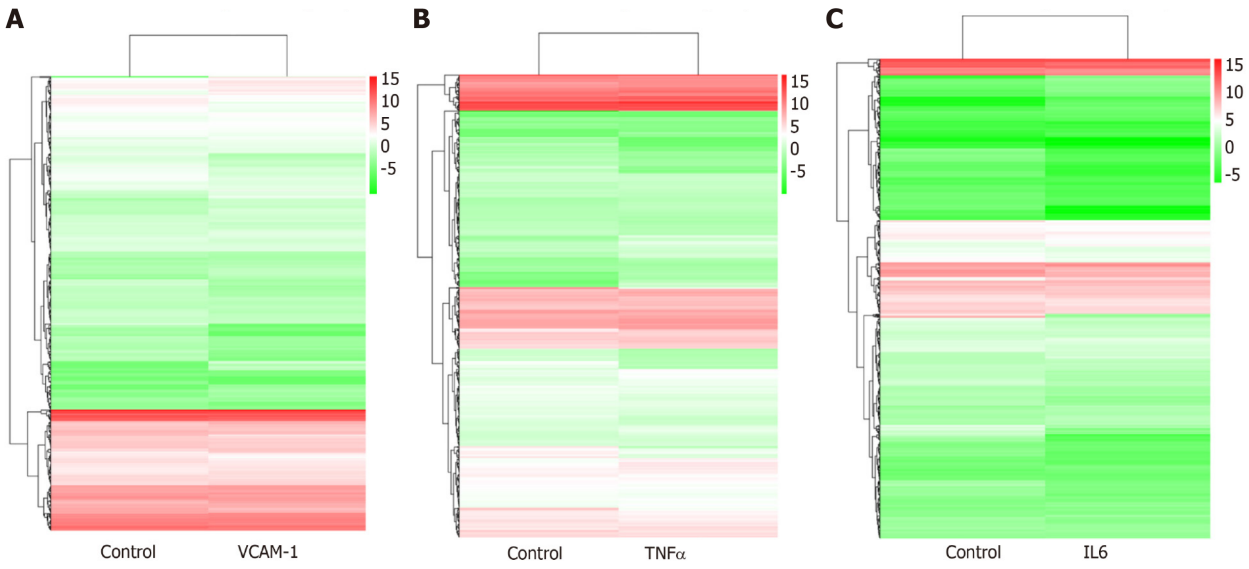
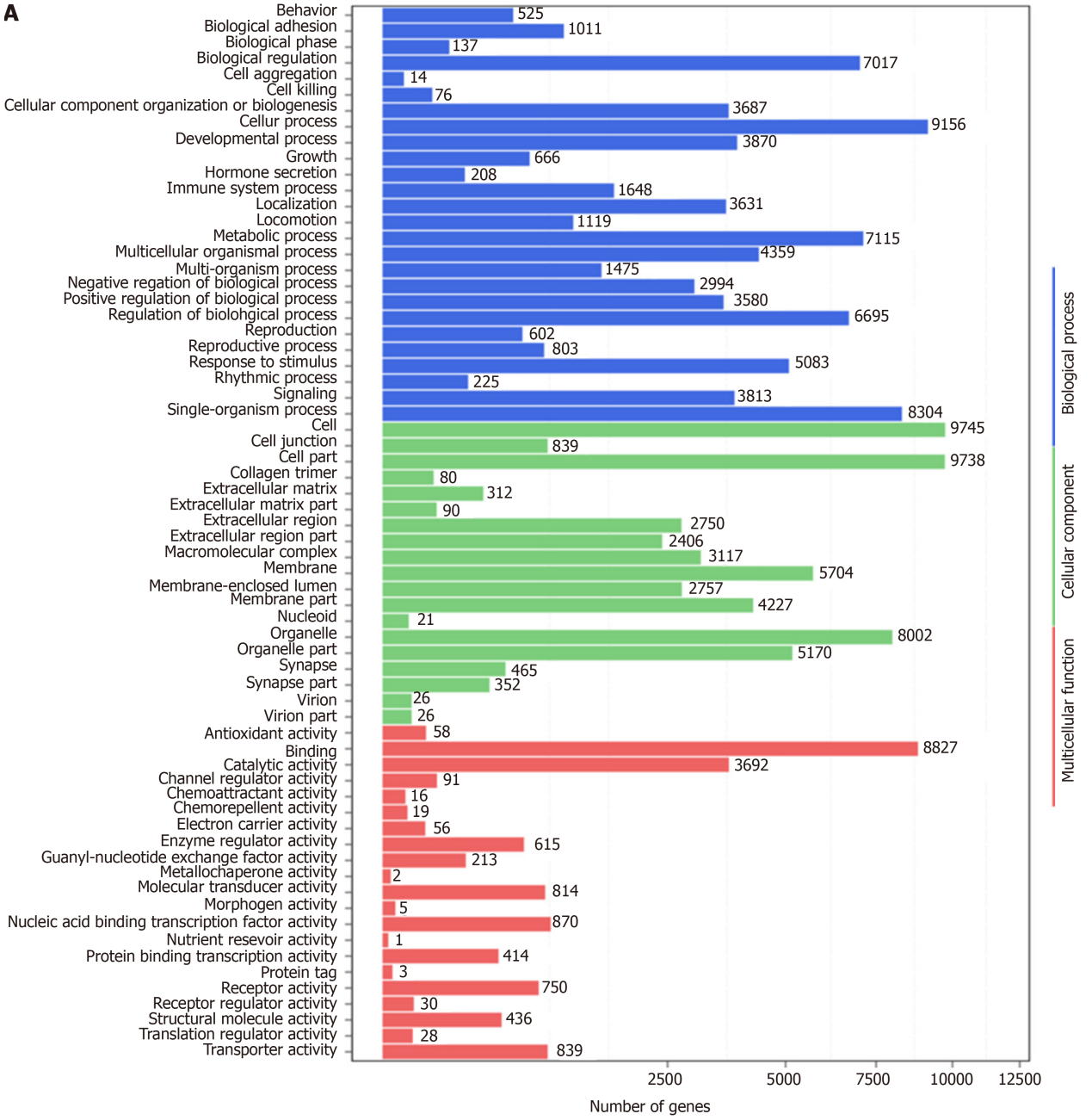


Figure 7 Hierarchical clustering of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group *vs* control group; B: Tumor necrosis factor α group *vs* control group; C: Interleukin 6 group *vs* control group. The X axis represents each pair of differences, and the Y axis represents differentially expressed miRNAs. The colors indicate the fold change, with red showing up-regulation, and blue showing down-regulation. TNF: Tumor necrosis factor; IL6: Interleukin 6.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Behavior 525
Biological adhesion 1011
Biological phase 137
Biological regulation 7017
Cell aggregation 14
Cell killing 76
Cellular component organization or biologenesis 3687
Cellur process 9156
Developmental process 3870
Growth 666
Hormone secretion 208
Immune system process 1648
Localization 3631
Locomotion 1119
Metabolic process 7115
Multicellular organismal process 4359
Multi-organism process 1475
Negative regation of biological process 2994
Positive regulation of biological process 3580
Regulation of biolohgical process 6695
Reproduction 602
Reproductive process 803
Response to stimulus 5083
Rhythmic process 225
Signaling 3813
Single-organism process 8304
Cell 9745
Cell junction 839
Cell part 9738
Collagen trimer 80
Extracellular matrix 312
Extracellular matrix part 90
Extracellular region 2750
Extracellular region part 2406
Macromolecular complex 3117
Membrane 5704
Membrane-enclosed lumen 2757
Membrane part 4227
Nucleoid 21
Organelle 8002
Organelle part 5170
Synapse 465
Synapse part 352
Virion 26
Virion part 26
Antioxidant activity 58
Binding 8827
Catalytic activity 3692
Channel regulator activity 91
Chemoattractant activity 16
Chemorepellent activity 19
Electron carrier activity 56
Enzyme regulator activity 615
Guanyl-nucleotide exchange factor activity 213
Metallochaperone activity 2
Molecular transducer activity 814
Morphogen activity 5
Nucleic acid binding transcription factor activity 870
Nutrient resevoir activity 1
Protein binding transcription activity 414
Protein tag 3
Receptor activity 750
Receptor regulator activity 30
Structural molecule activity 436
Translation regulator activity 28
Transporter activity 839
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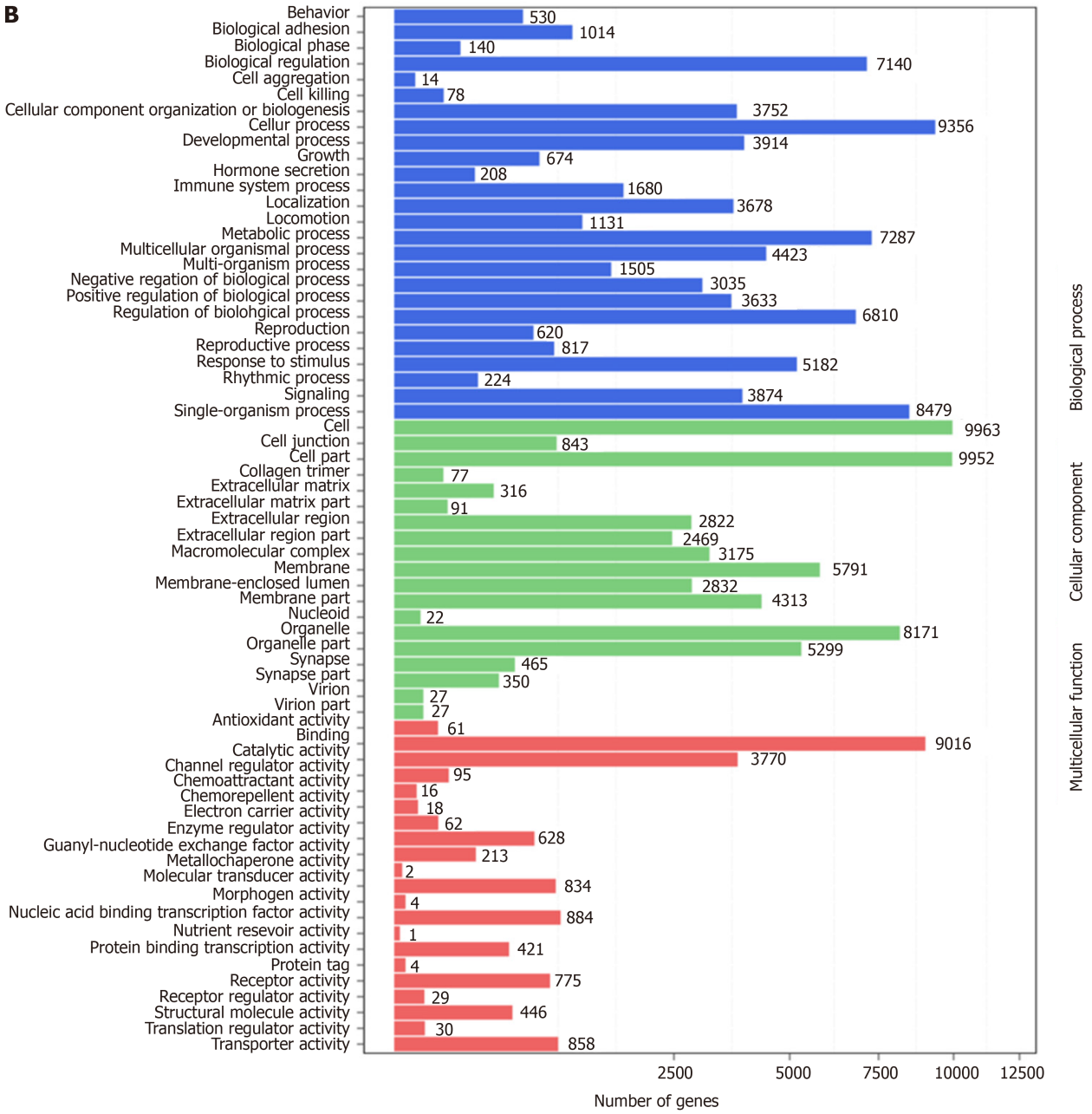
5000
7500
10000
12500
Number of genes
Biological process
Cellular component
Multicellular function


B
Behavior 530
Biological adhesion 1014
Biological phase 140
Biological regulation 7140
Cell aggregation 14
Cell killing 78
Cellular component organization or biologenesis 3752
Cellur process 9356
Developmental process 3914
Growth 674
Hormone secretion 208
Immune system process 1680
Localization 3678
Locomotion 1131
Metabolic process 7287
Multicellular organismal process 4423
Multi-organism process 1505
Negative regation of biological process 3035
Positive regulation of biological process 3633
Regulation of biolohgical process 6810
Reproduction 620
Reproductive process 817
Response to stimulus 5182
Rhythmic process 224
Signaling 3874
Single-organism process 8479
Cell 9963
Cell junction 843
Cell part 9952
Collagen trimer 77
Extracellular matrix 316
Extracellular matrix part 91
Extracellular region 2822
Extracellular region part 2469
Macromolecular complex 3175
Membrane 5791
Membrane-enclosed lumen 2832
Membrane part 4313
Nucleoid 22
Organelle 8171
Organelle part 5299
Synapse 465
Synapse part 350
Virion 27
Virion part 27
Antioxidant activity 61
Binding 9016
Catalytic activity 3770
Channel regulator activity 95
Chemoattractant activity 16
Chemorepellent activity 18
Electron carrier activity 62
Enzyme regulator activity 628
Guanyl-nucleotide exchange factor activity 213
Metallochaperone activity 2
Molecular transducer activity 834
Morphogen activity 4
Nucleic acid binding transcription factor activity 884
Nutrient resevoir activity 1
Protein binding transcription activity 421
Protein tag 4
Receptor activity 775
Receptor regulator activity 29
Structural molecule activity 446
Translation regulator activity 30
Transporter activity 858
2500
5000
7500
10000
12500
Number of genes
Biological process
Cellular component
Multicellular function

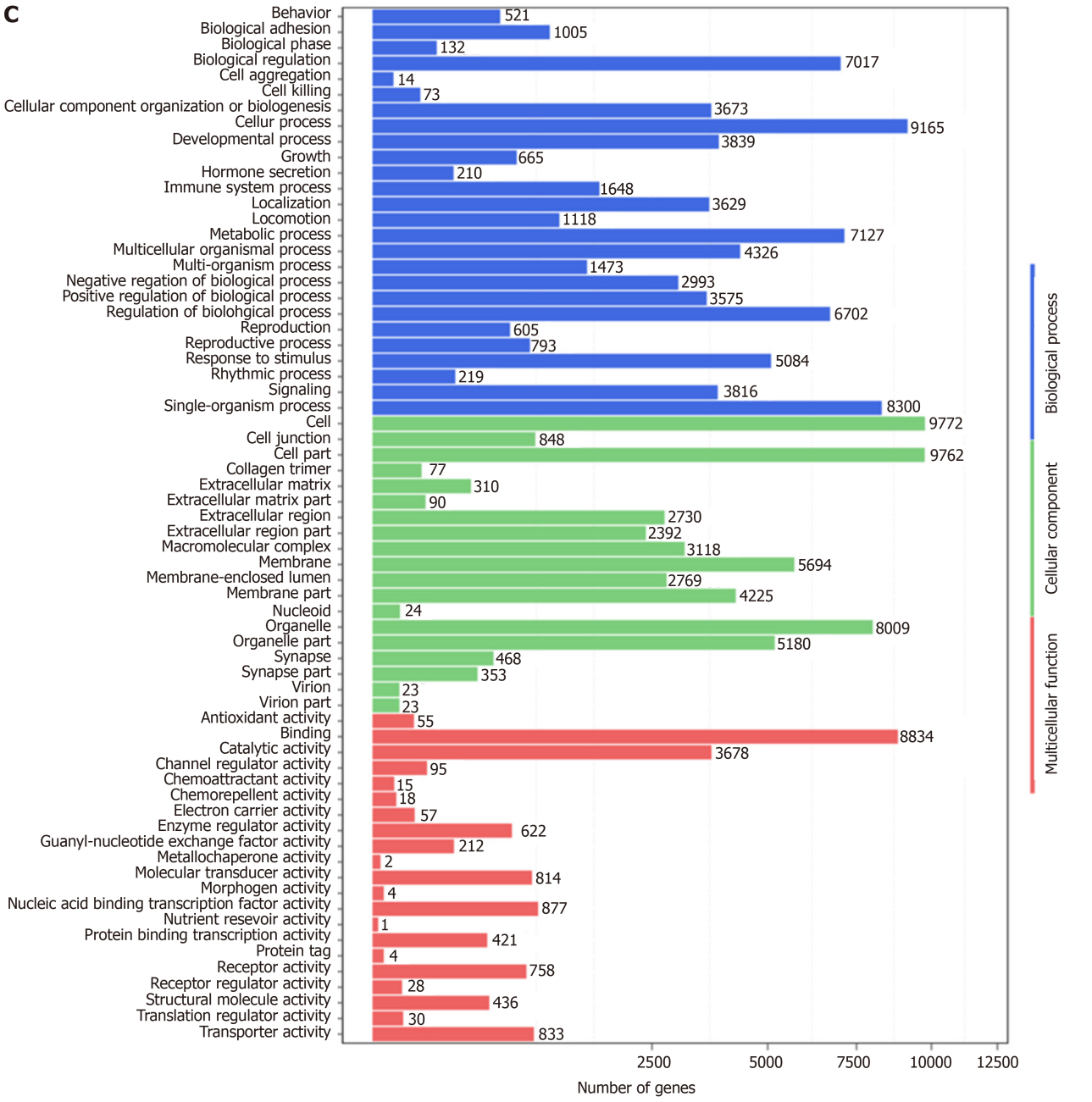
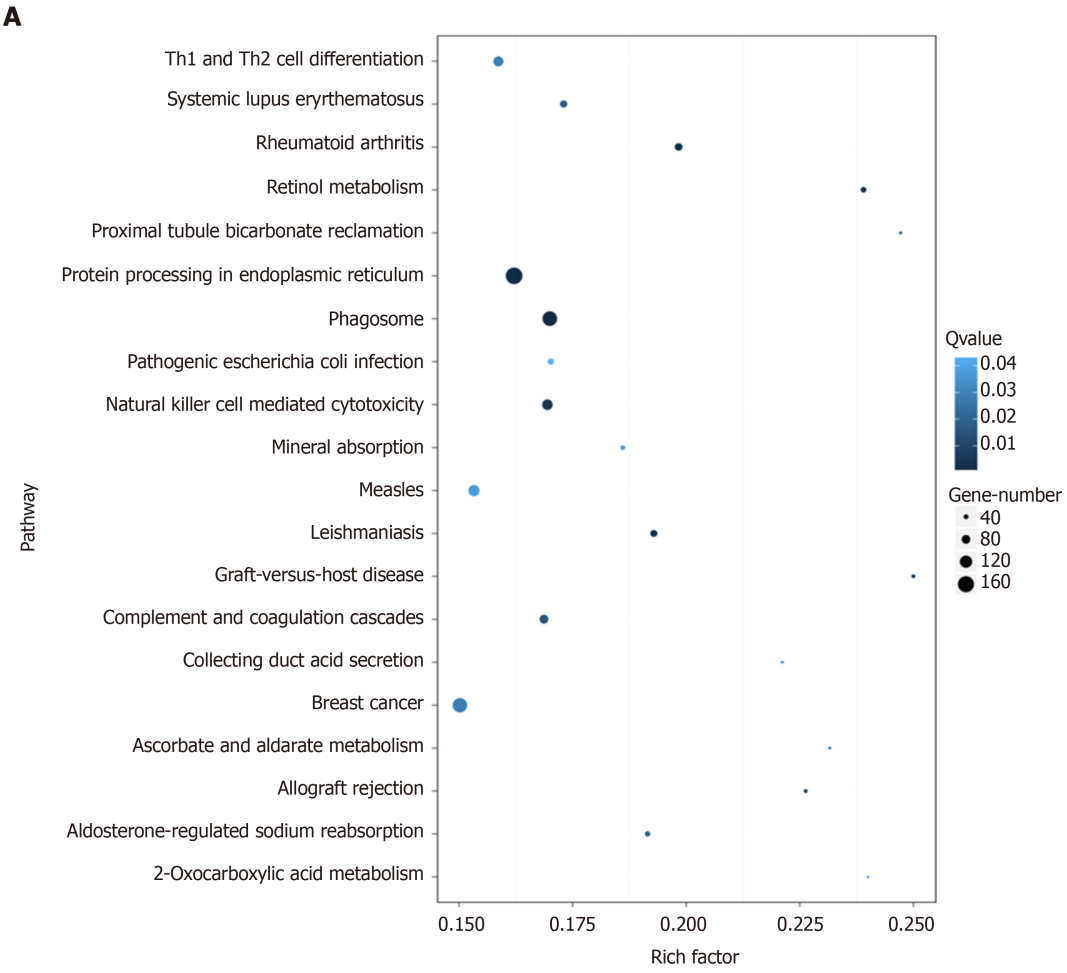


Figure 8 Gene ontology enrichment analysis of gene targets of differentially expressed miRNAs. A: Vascular cell adhesion molecule-1 group *vs* control group; B: Tumor necrosis factor α group *vs* control group; C: Interleukin 6 group *vs* control group. The X axis shows the number of differently expressed genes (their square root value), and the Y axis shows GO terms. All GO terms are grouped into three ontologies: Blue indicates biological process, brown indicates cellular components, and red indicates molecular function

A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Systemic lupus eryrthematosus
Rheumatoid arthritis
Retinol metabolism
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
Phagosome
Pathogenic escherichia coli infection
Natural killer cell mediated cytotoxicity
Mineral absorption
Measles
Leishmaniasis
Graft-versus-host disease
Complement and coagulation cascades
Collecting duct acid secretion
Breast cancer
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
2-Oxocarboxylic acid metabolism
Pathway
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0.175
0.200
0.225
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Qvalue
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0.03
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0.01
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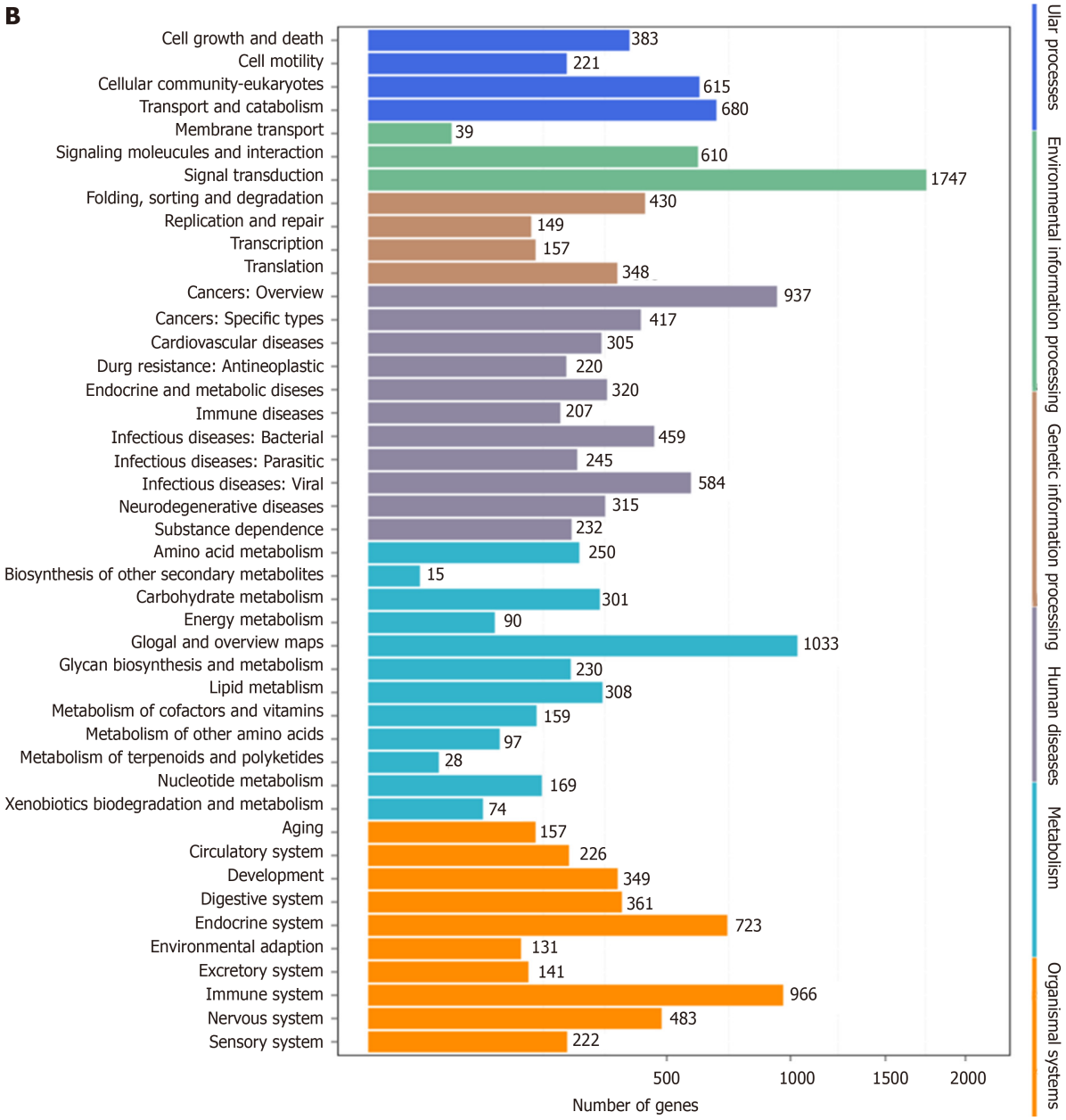
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Cell growth and death 383
Cell motility 221
Cellular community-eukaryotes 615
Transport and catabolism 680
Membrane transport 39
Signaling moleucules and interaction 610
Signal transduction 1747
Folding, sorting and degradation 430
Replication and repair 149
Transcription 157
Translation 348
Cancers: Overview 937
Cancers: Specific types 417
Cardiovascular diseases 305
Durg resistance: Antineoplastic 220
Endocrine and metabolic diseses 320
Immune diseases 207
Infectious diseases: Bacterial 459
Infectious diseases: Parasitic 245
Infectious diseases: Viral 584
Neurodegenerative diseases 315
Substance dependence 232
Amino acid metabolism 250
Biosynthesis of other secondary metabolites 15
Carbohydrate metabolism 301
Energy metabolism 90
Glogal and overview maps 1033
Glycan biosynthesis and metabolism 230
Lipid metablism 308
Metabolism of cofactors and vitamins 159
Metabolism of other amino acids 97
Metabolism of terpenoids and polyketides 28
Nucleotide metabolism 169
Xenobiotics biodegradation and metabolism 74
Aging 157
Circulatory system 226
Development 349
Digestive system 361
Endocrine system 723
Environmental adaption 131
Excretory system 141
Immune system 966
Nervous system 483
Sensory system 222
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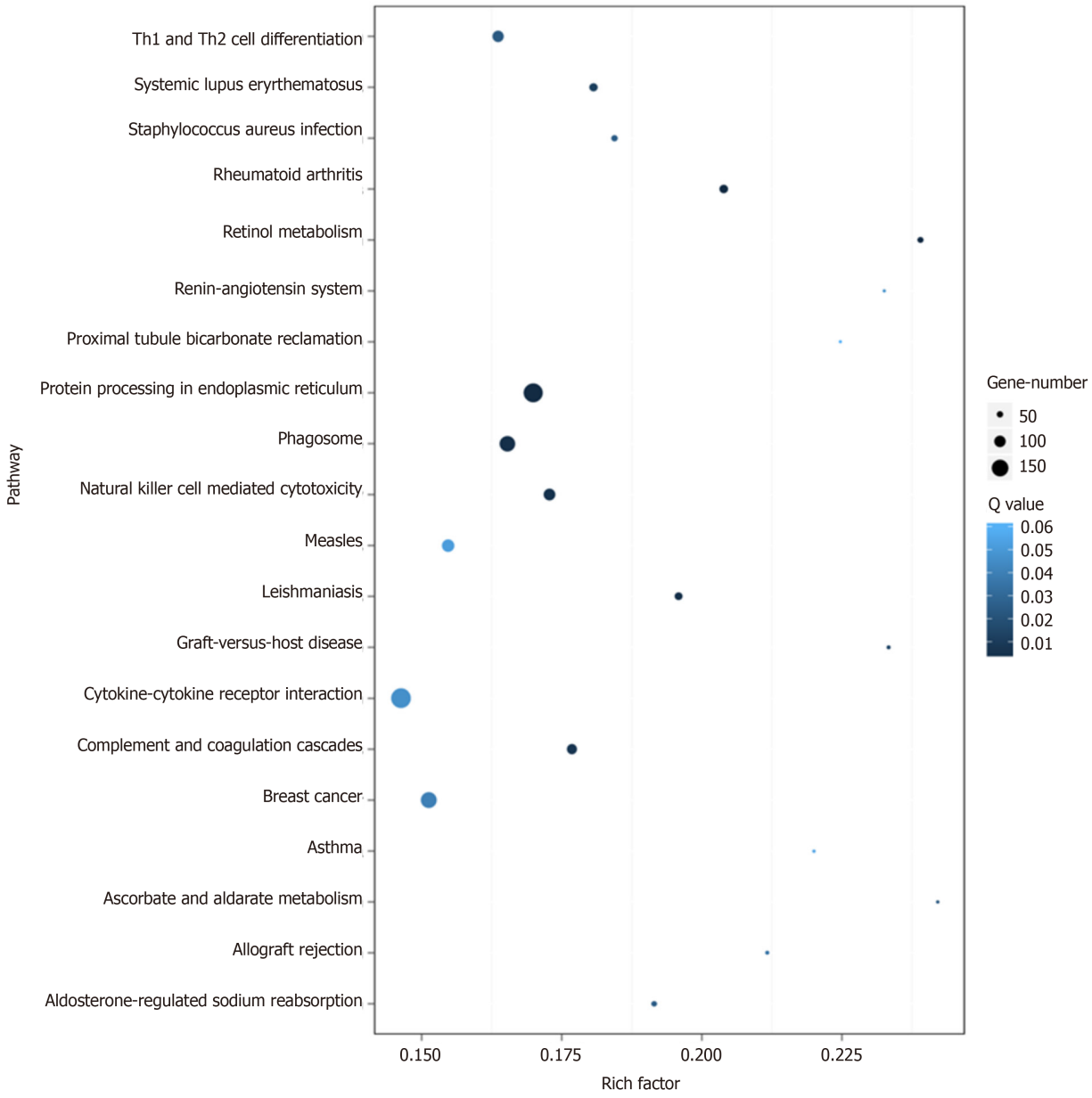
1000
1500
2000
Number of genes
Ular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems

C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Systemic lupus eryrthematosus
Staphylococcus aureus infection
Rheumatoid arthritis
Retinol metabolism
Renin-angiotensin system
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
Phagosome
Natural killer cell mediated cytotoxicity
Measles
Leishmaniasis
Graft-versus-host disease
Cytokine-cytokine receptor interaction
Complement and coagulation cascades
Breast cancer
Asthma
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
Pathway
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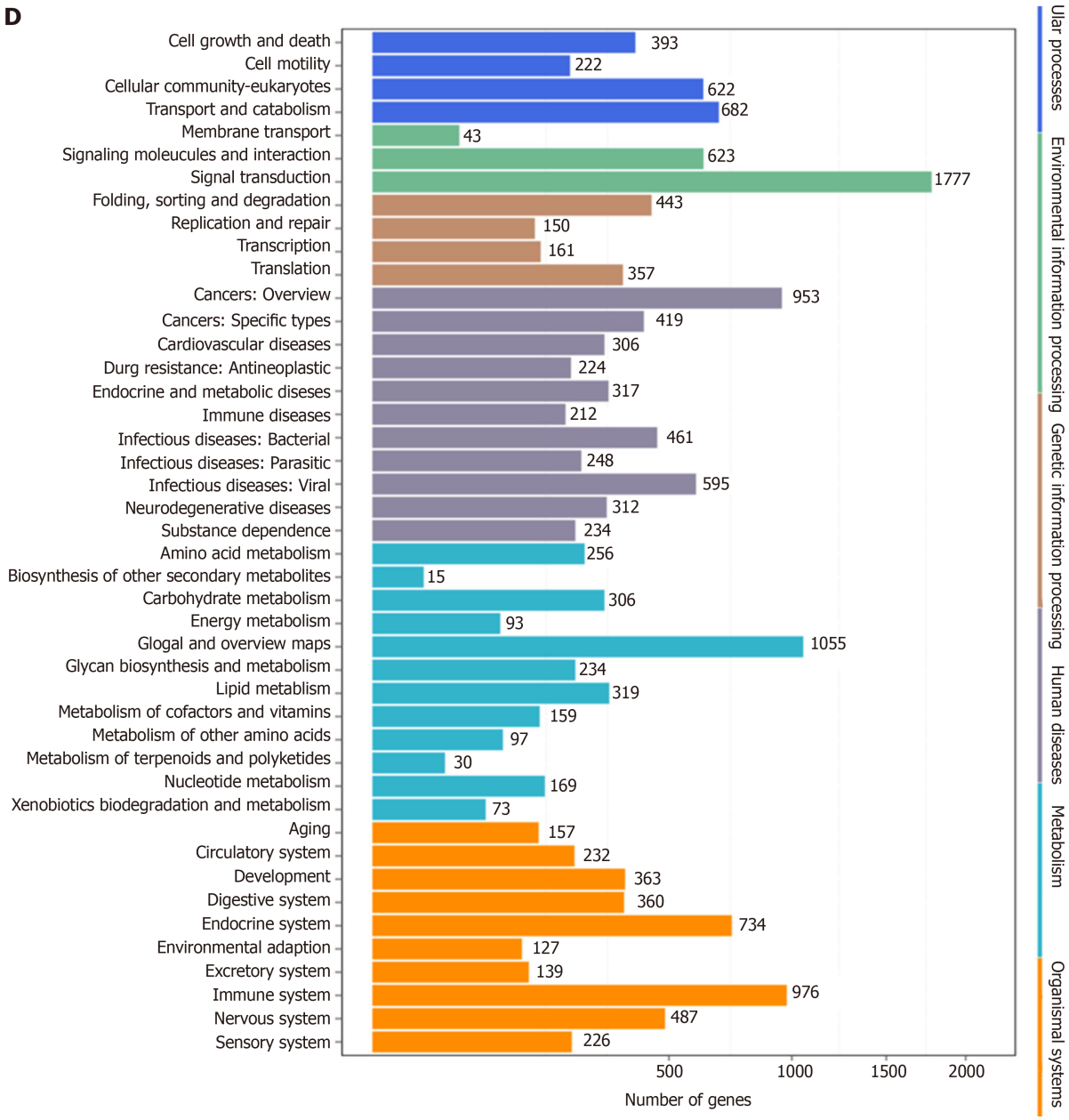
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Signal transduction 1777
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Infectious diseases: Bacterial 461
Infectious diseases: Parasitic 248
Infectious diseases: Viral 595
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Carbohydrate metabolism 306
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Metabolism of terpenoids and polyketides 30
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Aging 157
Circulatory system 232
Development 363
Digestive system 360
Endocrine system 734
Environmental adaption 127
Excretory system 139
Immune system 976
Nervous system 487
Sensory system 226
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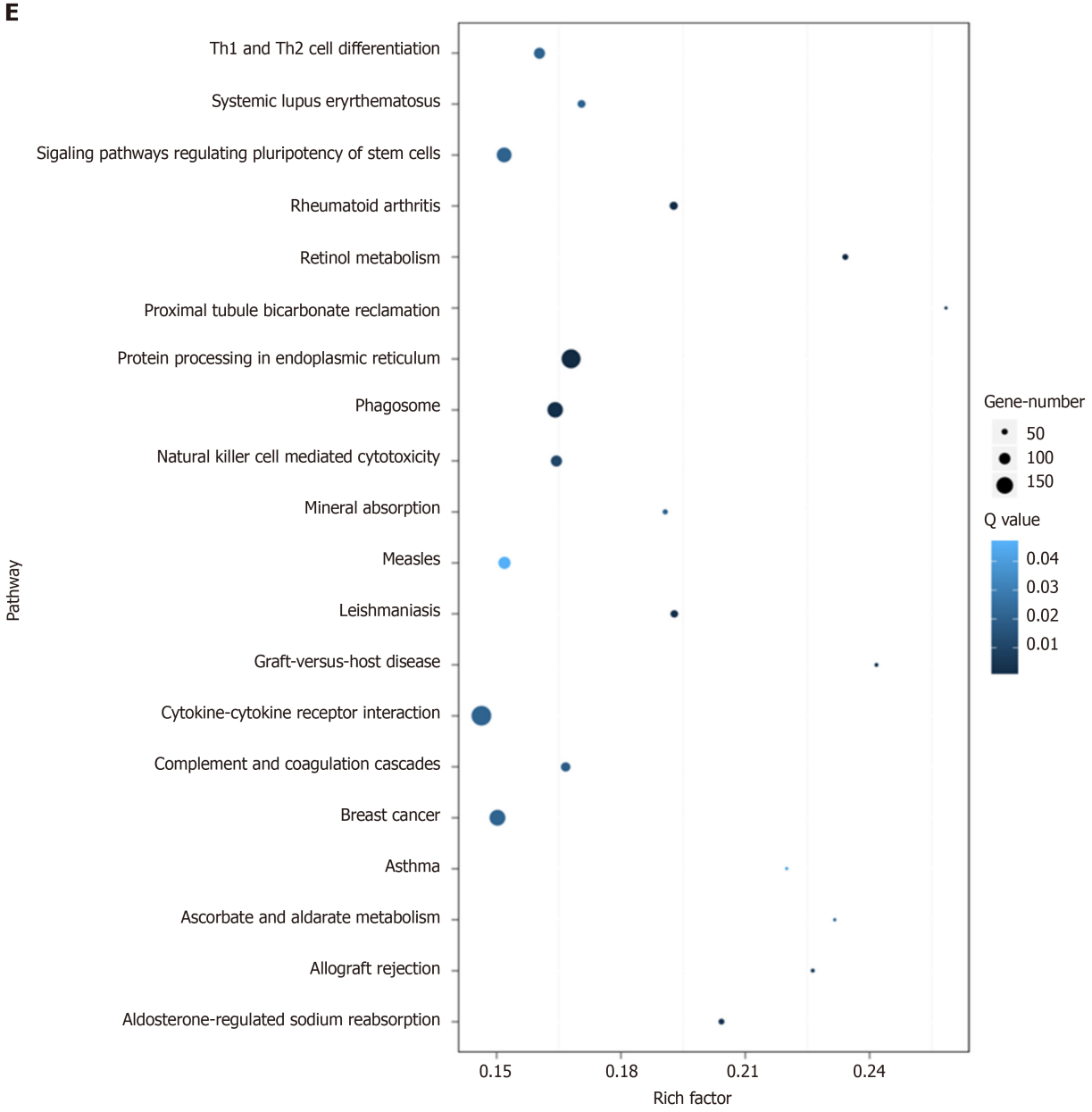
1000
1500
2000
Number of genes
Ular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems

E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Systemic lupus eryrthematosus
Sigaling pathways regulating pluripotency of stem cells
Rheumatoid arthritis
Retinol metabolism
Proximal tubule bicarbonate reclamation
Protein processing in endoplasmic reticulum
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Natural killer cell mediated cytotoxicity
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Cytokine-cytokine receptor interaction
Complement and coagulation cascades
Breast cancer
Asthma
Ascorbate and aldarate metabolism
Allograft rejection
Aldosterone-regulated sodium reabsorption
Pathway
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Gene-number
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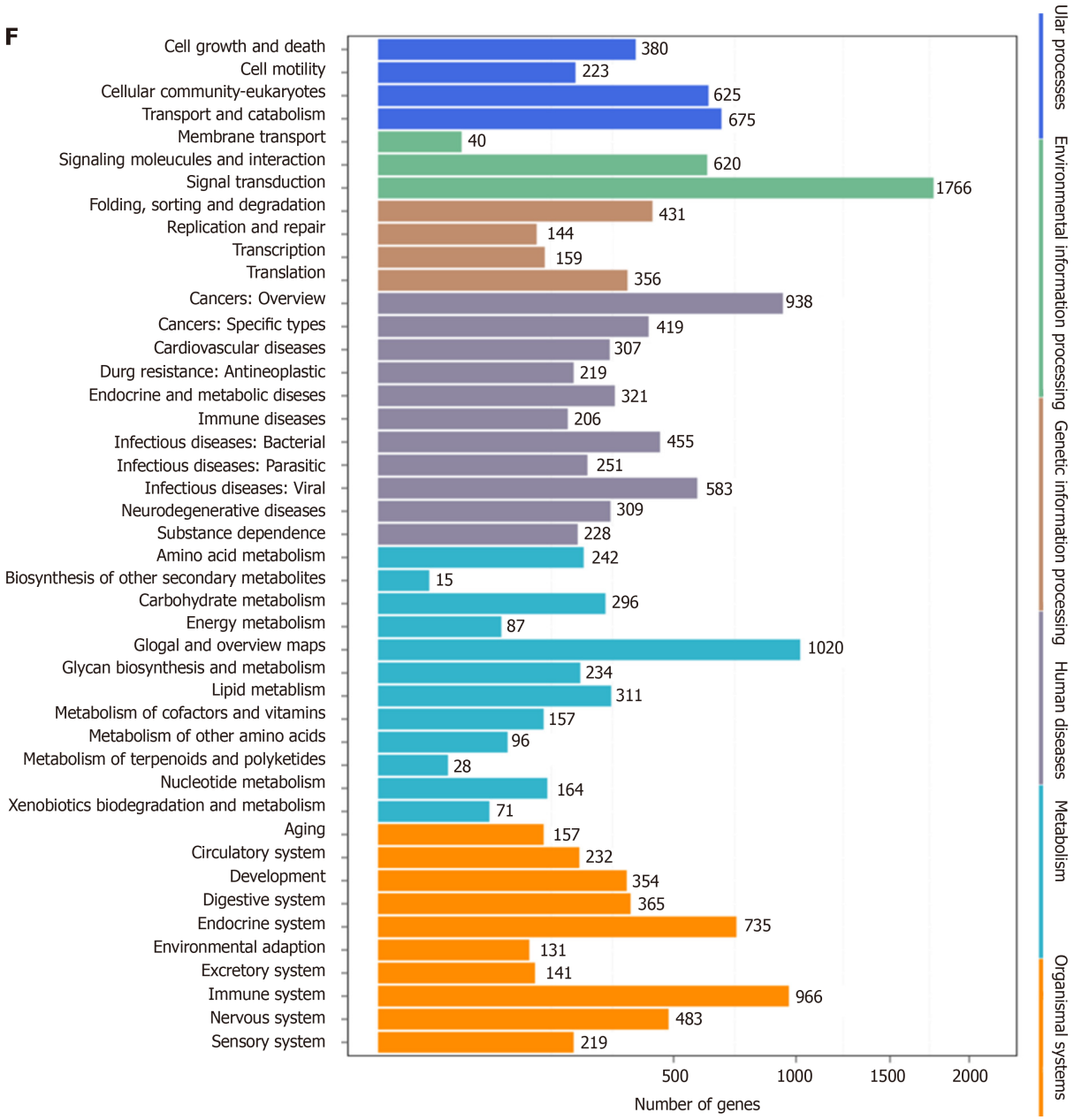
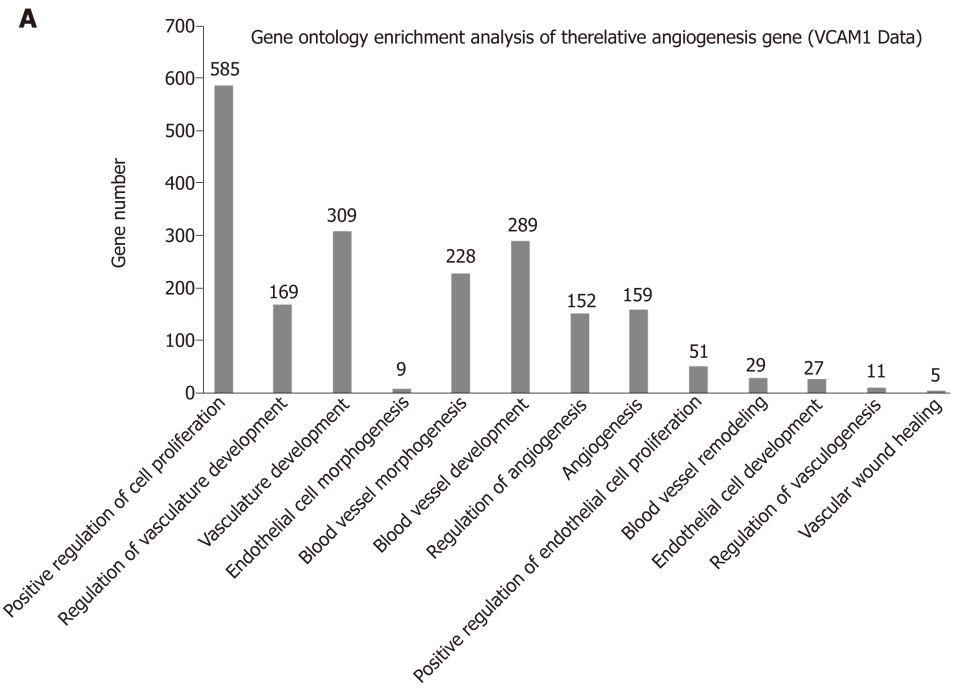


Figure 9 Pathway enrichment analysis of gene targets of differentially expressed miRNAs. A: Comparison of the top 20 enriched pathway terms between vascular cell adhesion molecule-1 (VCAM-1) group and control group; B: Comparison of the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification between VCAM-1 group and control group; C: Comparison of the top 20 enriched pathway terms between tumor necrosis factor (TNF) α group and control group; D: Comparison of the KEGG classification between TNFα group and control group; E: Comparison of the top 20 enriched pathway terms between interleukin 6 (IL6) group and control group; F: Comparison of the KEGG classification between IL6 group and control group. A, C, and E: The top 20 enriched pathway terms displayed as scatterplots. The rich factor is the ratio of target gene numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the rich factor, the greater the degree of enrichment. The Q-value is the corrected *P*-value and ranges from 0-1; the lower the Q-value, the greater the level of enrichment; B, D, and F: The X axis shows the number of target genes, and the Y axis shows the second KEGG pathway terms. The first pathway terms are indicated using different colors. The second pathway terms are subgroups of the first pathway terms and are grouped together on the X axis on the right side.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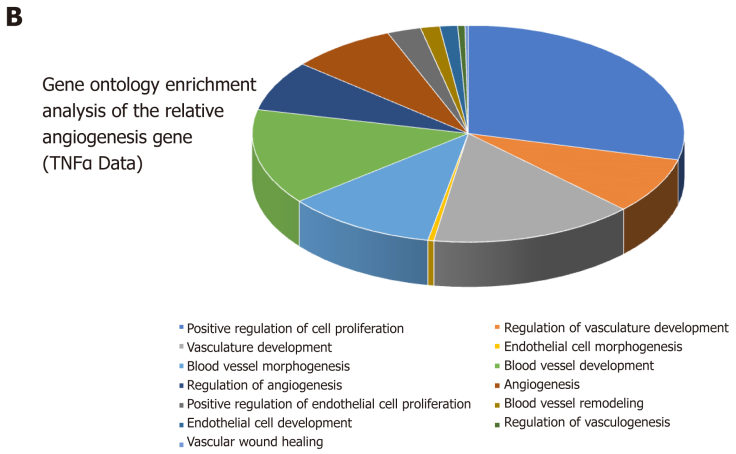
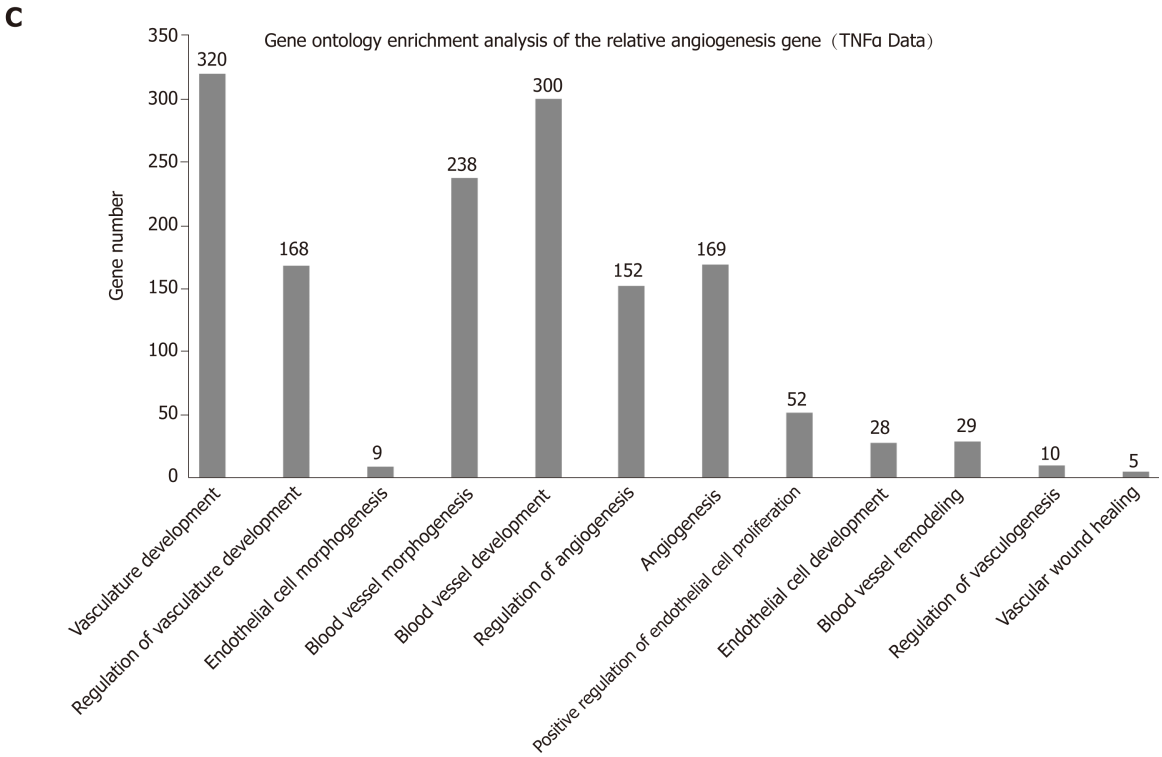
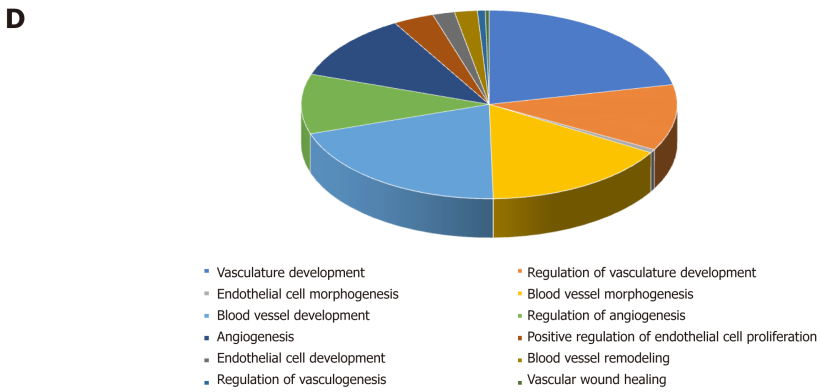
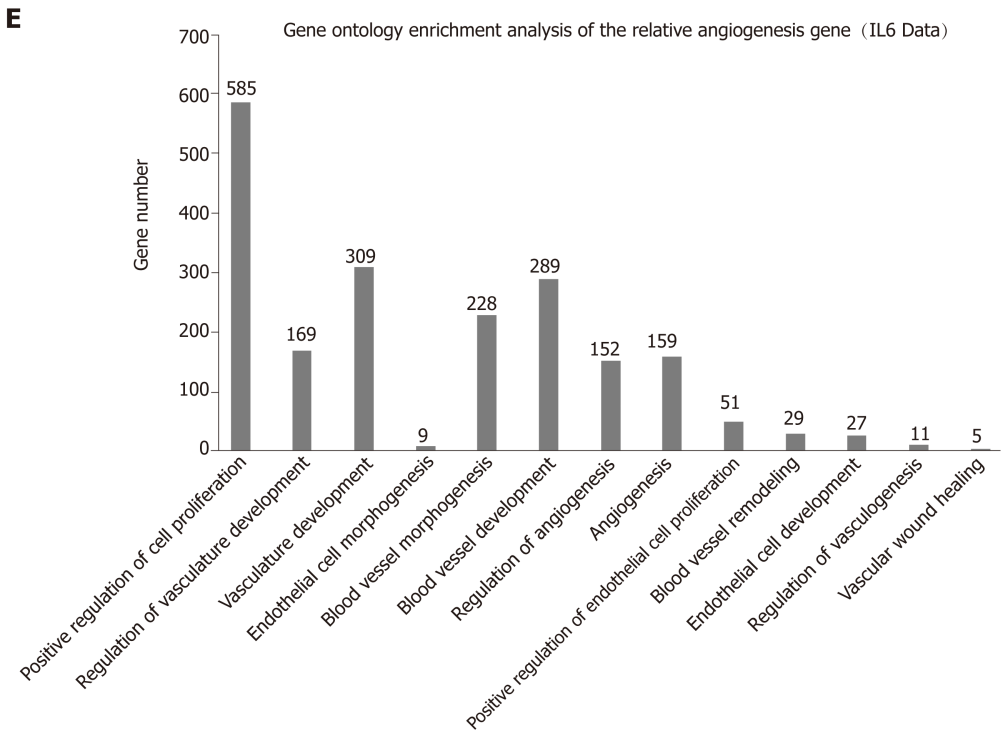
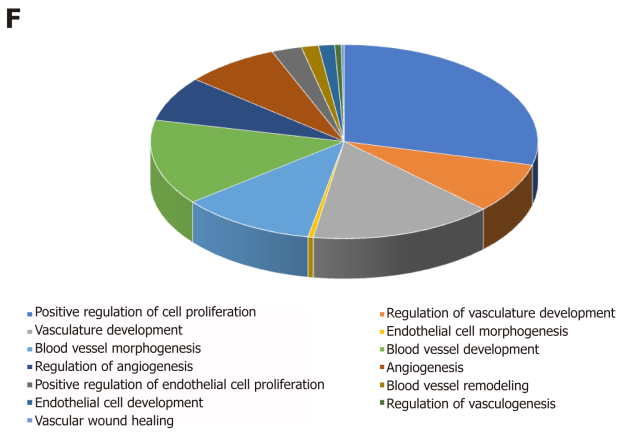
Gene ontology enrichment analysis of therelative angiogenesis gene (VCAM1 Data)
Gene number
700
600
500
400
300
200
100
0
585
169
309
9
228
289
152
159
51
29
27
11
5
Positive regulation of cell proliferation
Regulation of vasculature development
Vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Blood vessel remodeling
Endothelial cell development
Regulation of vasculogenesis
Vascular wound healing

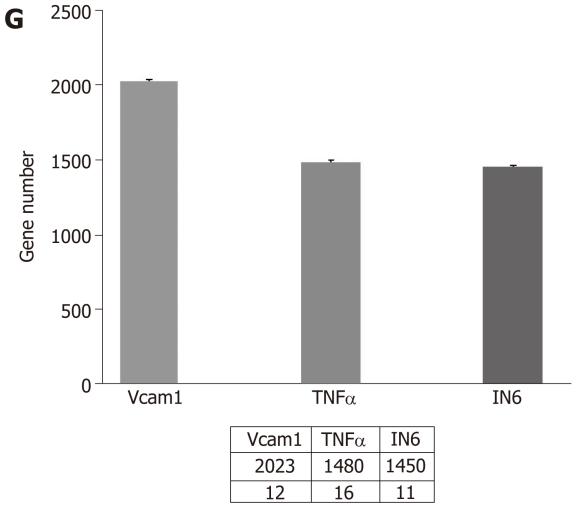

B
Gene ontology enrichment analysis of the relative angiogenesis gene (TNFα Data)
Positive regulation of cell proliferation
Regulation of vasculature development
Vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Blood vessel remodeling
Endothelial cell development
Regulation of vasculogenesis
Vascular wound healing


C
Gene ontology enrichment analysis of the relative angiogenesis gene（TNFα Data）
Gene number
350
300
250
200
150
100
50
0
320
168
9
238
300
152
169
52
28
29
10
5
Vasculature development
Regulation of vasculature development
Endothelial cell morphogenesis
Blood vessel morphogenesis
Blood vessel development
Regulation of angiogenesis
Angiogenesis
Positive regulation of endothelial cell proliferation
Endothelial cell development
Blood vessel remodeling
Regulation of vasculogenesis
Vascular wound healing



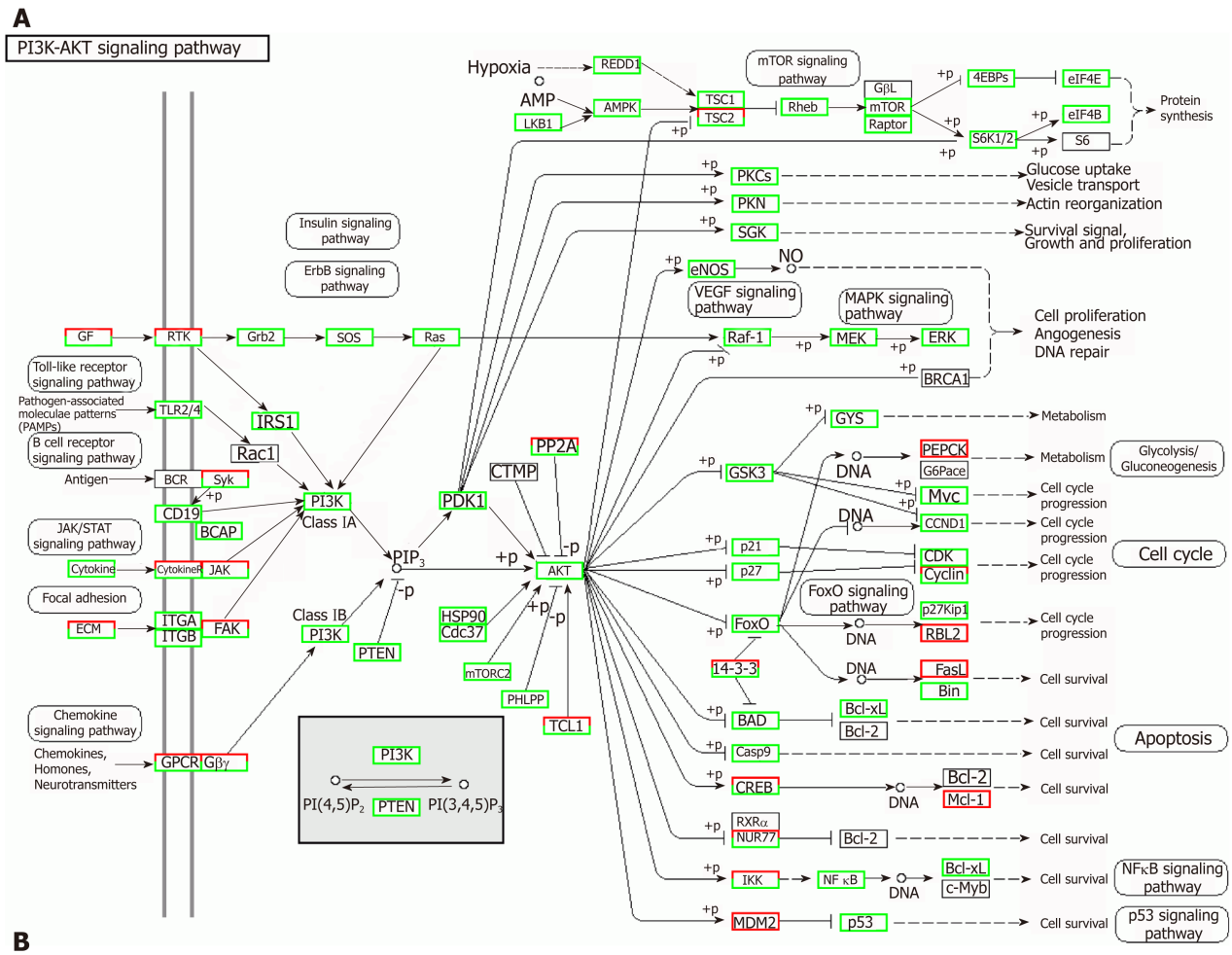






Vcam1	TNFα	IN6
2023	1480	1450
12	16	11

Figure 10 Gene ontology enrichment analysis of angiogenesis-related genes. A: The number of angiogenesis-related genes in the vascular cell adhesion molecule-1 (VCAM-1) group; B: The proportion of angiogenesis-related genes in the VCAM-1 group; C: The number of angiogenesis-related genes in the tumor necrosis factor (TNF) α group; D: The proportion of angiogenesis-related genes in the TNFα group; E: The number of angiogenesis gene distributions in the interleukin 6 (IL6) group; F: The proportion of angiogenesis-related genes in the IL6 group; G: Comparison of the number of angiogenesis-related genes in the three groups to that of the control group.


A
PI3K-AKT signaling pathway
Hypoxia
REDD1
AMP
LKB1
AMPK
mTOR signaling pathway
TSC1
TSC2
Rheb
GβL
mTOR
Raptor
4EBPs
eIF4E
eIF4B
S6K1/2
S6
Protein synthesis
PKCs
PKN
SGK
Glucose uptake
Vesicle transport
Actin reorganization
Survival signal,
Growth and proliferation
Insulin signaling pathway
ErbB signaling pathway
eNOS
NO
VEGF signaling pathway
MAPK signaling pathway
GF
RTK
Grb2
SOS
Ras
Raf-1
MEK
ERK
BRCA1
Cell proliferation
Angogenesis
DNA repair
Toll-like receptor signaling pathway
Pathogen-associated moleculae patterns (PAMPs)
TLR2/4
IRS1
Rac1
B cell receptor signaling pathway
Antigen
BCR
Syk
CD19
BCAP
PI3K
Class IA
PIP3
PDK1
PP2A
CTMP
AKT
JAK/STAT signaling pathway
Cytokine
CytokineR
JAK
Focal adhesion
ECM
ITGA
ITGB
FAK
Class IB
PI3K
PTEN
HSP90
Cdc37
mTORC2
PHLPP
TCL1
GYS
Metabolism
GSK3
DNA
PEPCK
G6Pace
Glycolysis/
Gluconeogenesis
Myc
CCND1
Cell cycle progression
p21
p27
CDK
Cyclin
Cell cycle
FoxO signaling pathway
FoxO
p27Kip1
RBL2
14-3-3
FasL
Bin
Cell survival
BAD
Bcl-xL
Bcl-2
Apoptosis
Casp9
CREB
Mcl-1
RXRα
NUR77
IKK
NF κB
c-Myb
NFκB signaling pathway
MDM2
p53
p53 signaling pathway
Chemokine signaling pathway
Chemokines, Homones, Neurotransmitters
GPCR
Gβγ
PI3K
PI(4,5)P2
PTEN
PI(3,4,5)P3
B
PI3K-AKT signaling pathway

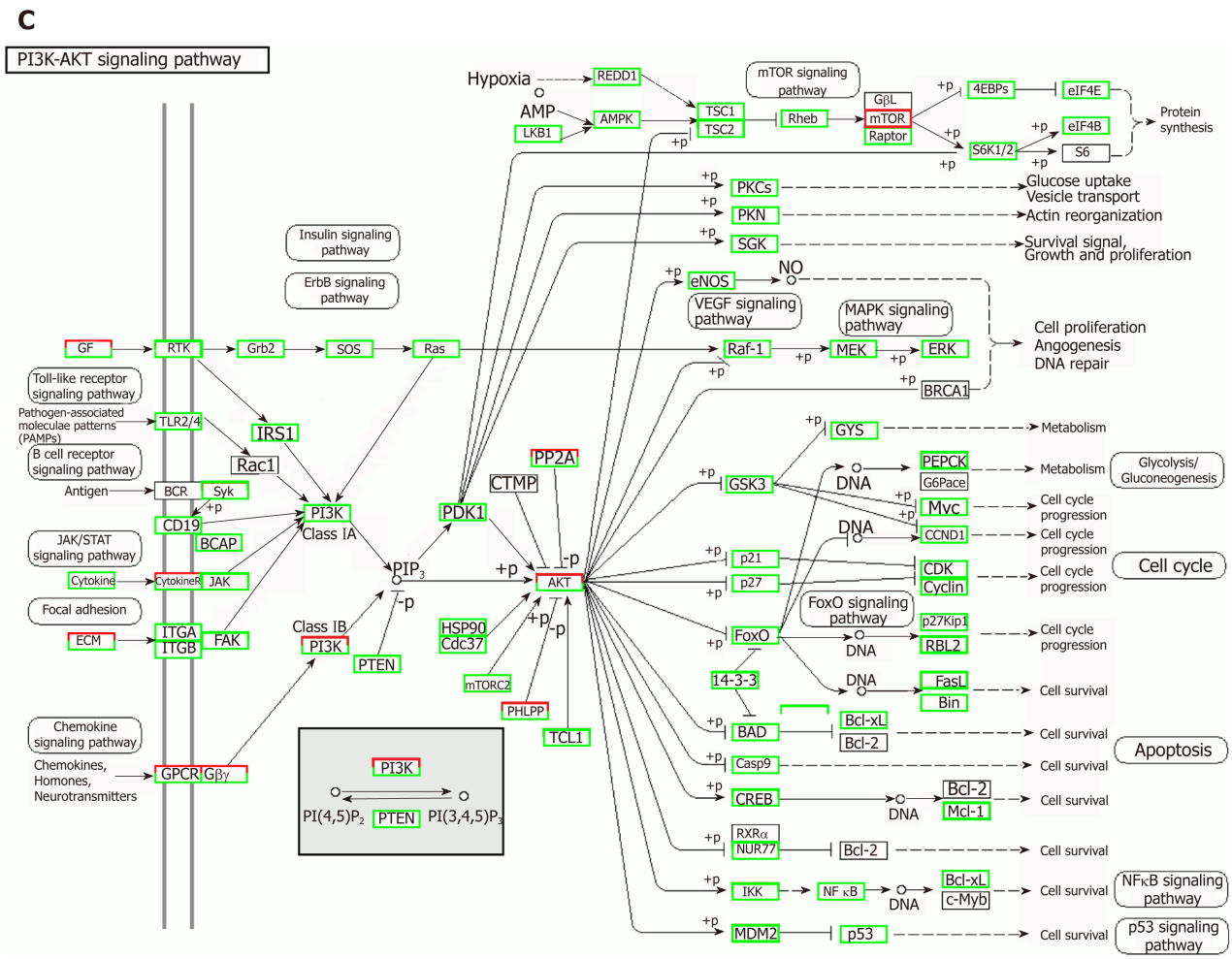
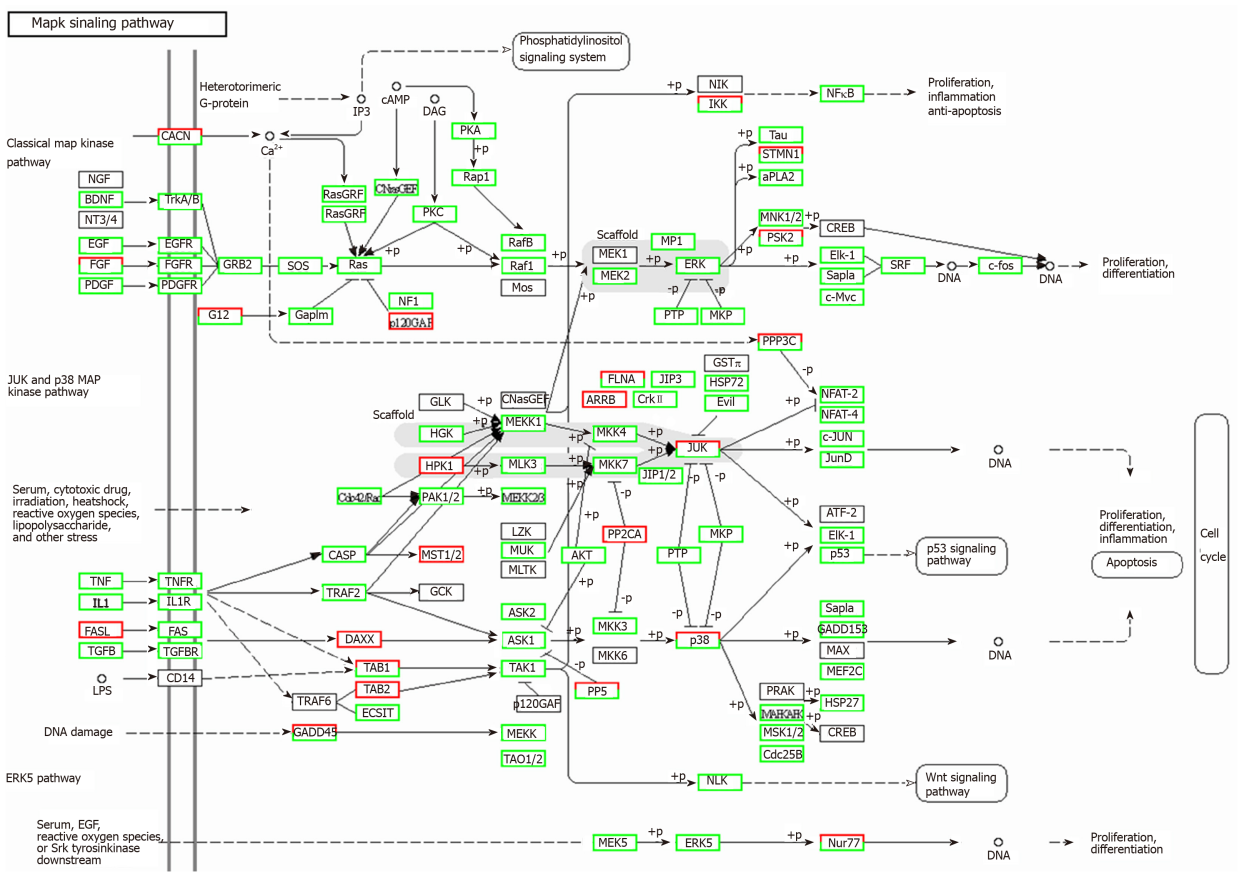
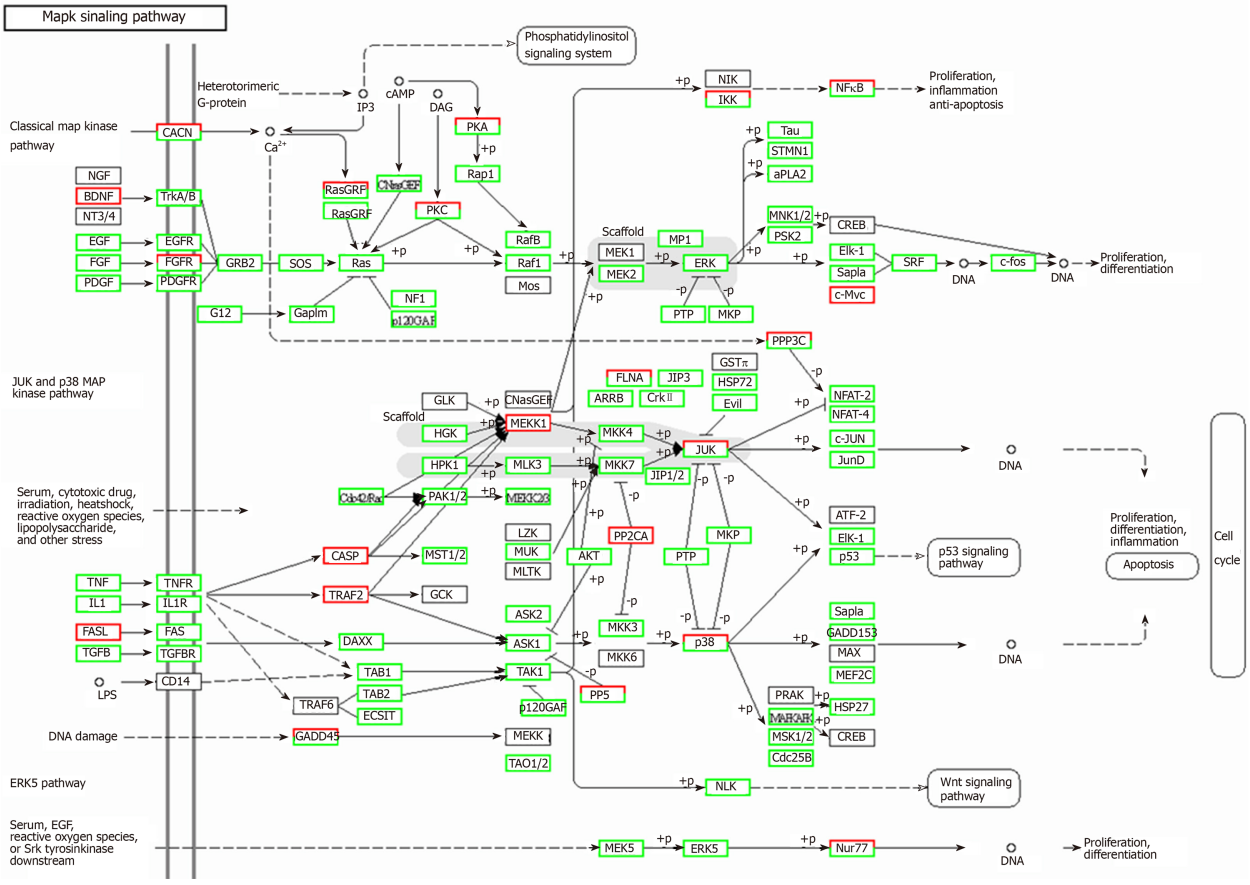


Figure 11 Regulatory mechanism of the PI3K-AKT signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

A


Mapk sinaling pathway
Phosphatidylinositol signaling system
Heterotorimeric G-protein
IP3
cAMP
DAG
Classical map kinase pathway
CACN
Ca2+
PKA
Rap1
RasGRF
PKC
NGF
BDNF
NT3/4
TrkA/B
EGF
FGF
PDGF
EGFR
FGFR
PDGFR
GRB2
SOS
Ras
RafB
Raf1
Mos
NF1
p120GAP
G12
Gaplm
Scaffold
MP1
MEK1
MEK2
ERK
PTP
MKP
NIK
IKK
NFκB
Proliferation, inflammation anti-apoptosis
Tau
STMN1
aPLA2
MNK1/2
PSK2
CREB
Elk-1
Sapla
c-Myc
SRF
DNA
c-fos
Proliferation, differentiation
PPP3C
JUK and p38 MAP kinase pathway
GSTπ
FLNA
JIP3
HSP72
ARRB
CrkII
Evil
GLK
HGK
HPK1
CNasGEF
MEKK1
MKK4
MKK7
MLK3
JUK
JIP1/2
NFAT-2
NFAT-4
c-JUN
JunD
Cdc42/Rac
PAK1/2
MEK3/6
LZK
MUK
MLTK
AKT
PP2CA
CASP
MST1/2
ATF-2
Elk-1
p53
p53 signaling pathway
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
Serum, cytotoxic drug, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
TNF
IL1
FASL
TGFB
TNFR
IL1R
FAS
TGFBR
CD14
LPS
TRAF2
GCK
ASK2
ASK1
DAXX
TAB1
TAB2
ECSIT
TAK1
TRAF6
PP5
MKK3
MKK6
p38
Sapla
GADD153
MAX
MEF2C
PRAK
MAPKAPK2
HSP27
MSK1/2
CREB
Cdc25B
DNA damage
GADD45
MEKK
TAO1/2
ERK5 pathway
NLK
Wnt signaling pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
MEK5
ERK5
Nur77

B


Mapk sinaling pathway
Phosphatidylinositol signaling system
Heterotorimeric G-protein
Classical map kinase pathway
Scaffold
JUK and p38 MAP kinase pathway
ERK5 pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
Proliferation, inflammation anti-apoptosis
Proliferation, differentiation
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
p53 signaling pathway
Wnt signaling pathway
DNA damage
Serum, cytotoxic drug, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress

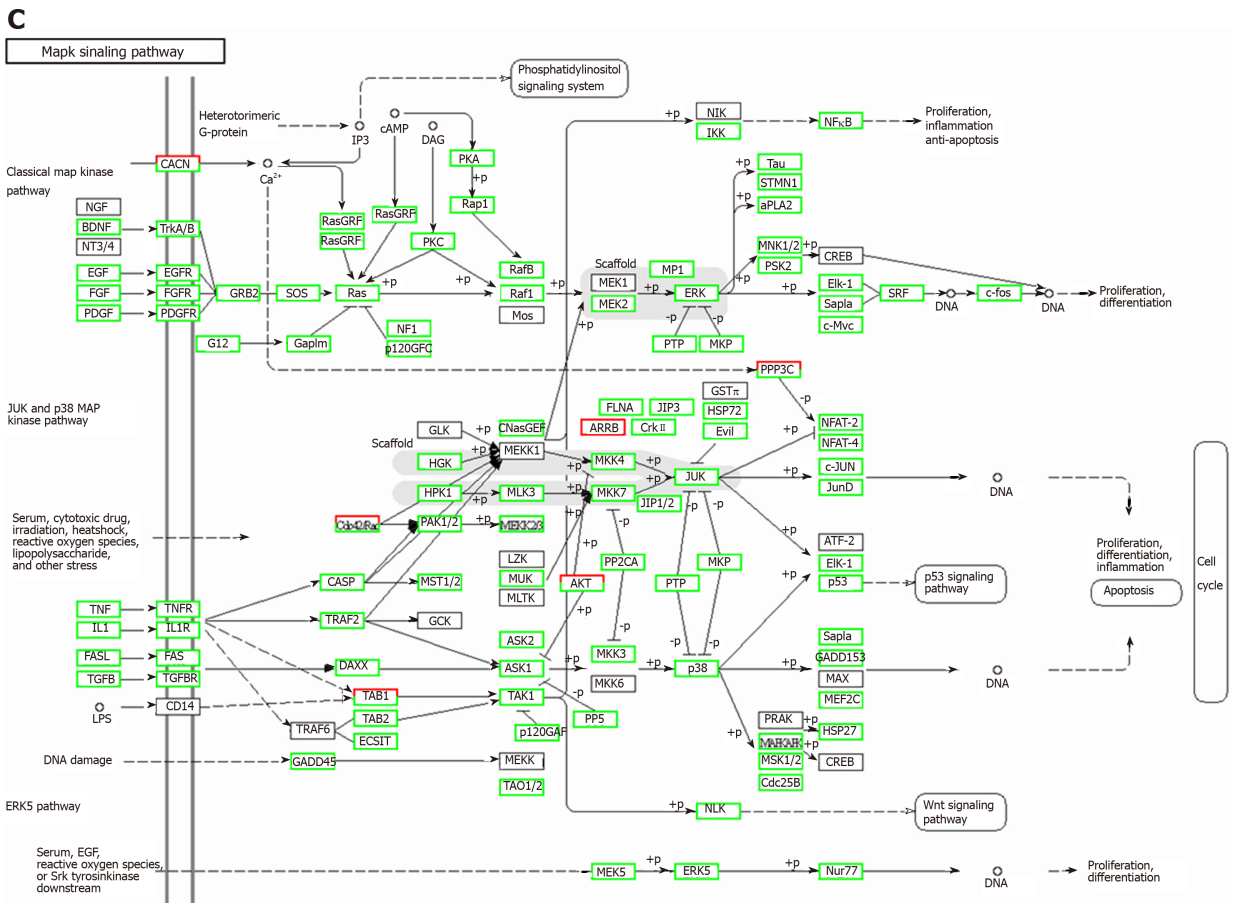


Figure 12 Regulatory mechanism of the MAPK signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

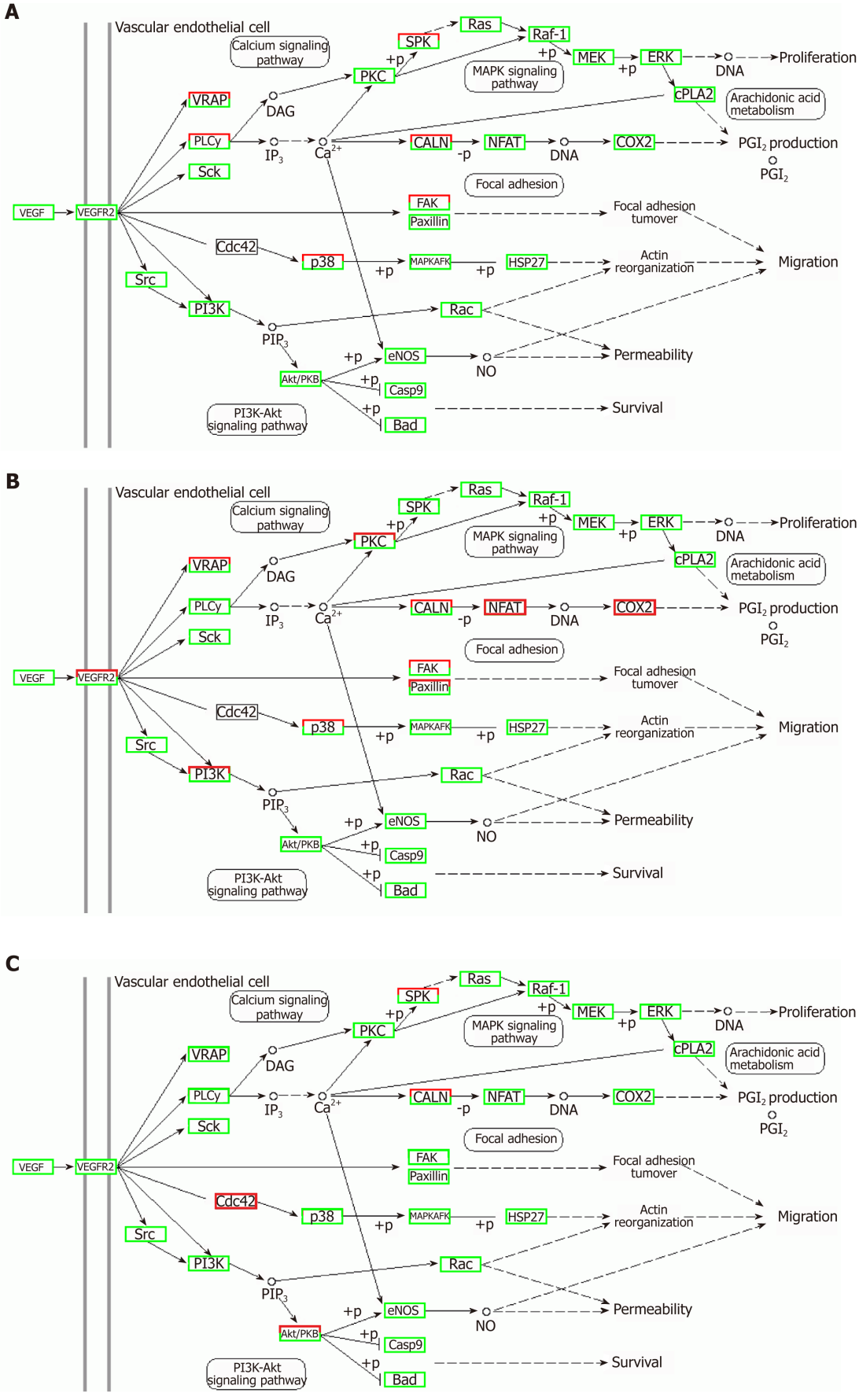


Figure 13 Regulatory mechanism of the VEGF signal pathway in different groups. A: Vascular cell adhesion molecule-1 group; B: Tumor necrosis factor α group; C: Interleukin 6 group. Up-regulated genes are marked with red borders and down-regulated genes with green borders. Unchanged genes are marked with black borders.

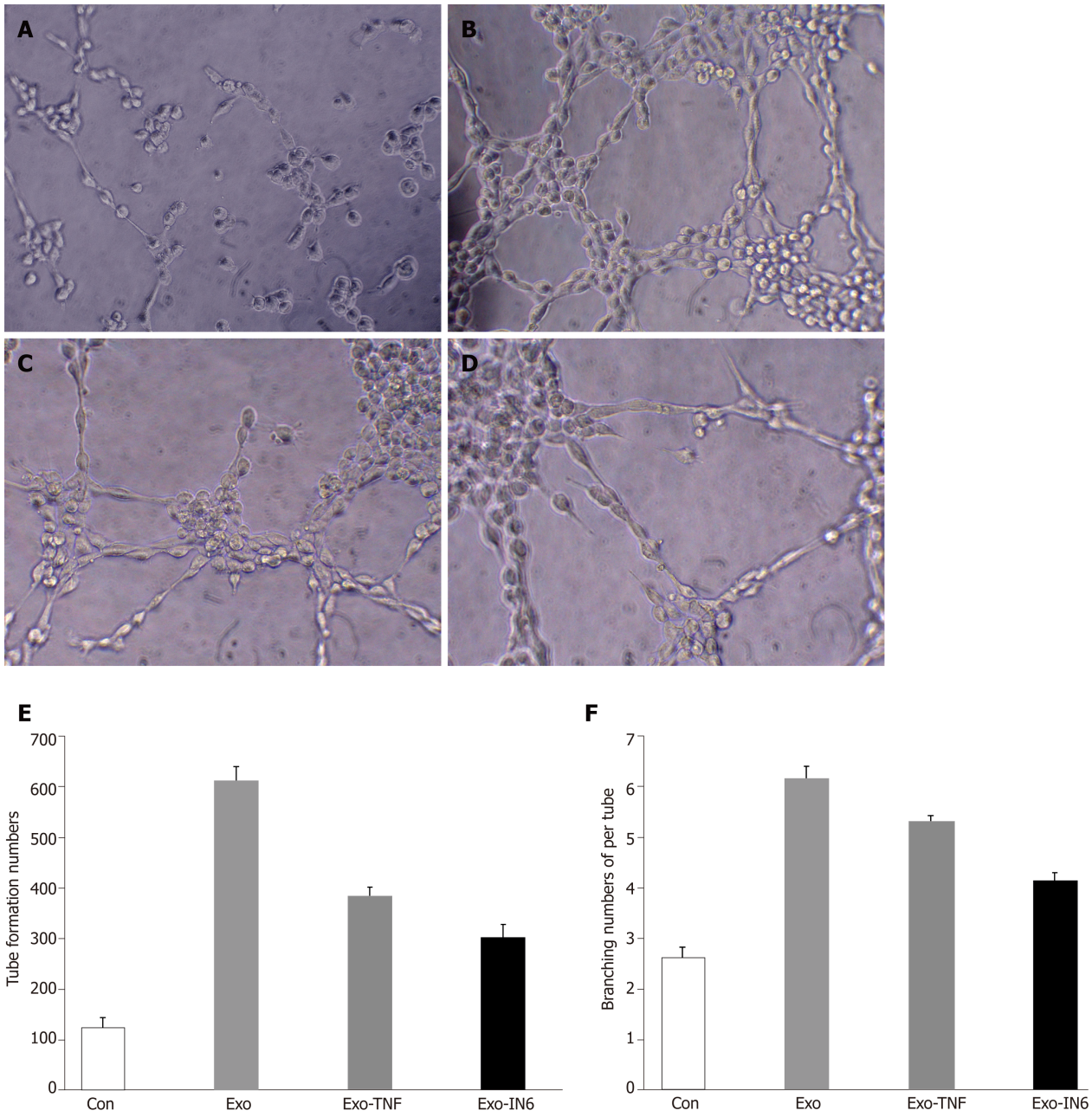


Figure 14 Tube formation in endothelial cells treated with mesenchymal stem cell exosomes. A: Micrograph showing human umbilical vein endothelial cells (HUVECs) cultured on Matrigel-coated plates in medium with phosphate buffered saline (control); B: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with mesenchymal stem cell exosomes (MSC-exo); C: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with MSCs-exo stimulated with tumor necrosis factor α; D: Micrograph showing HUVECs cultured on Matrigel-coated plates in medium with MSCs-exo stimulated with interleukin 6; E: The number of tubes formed in each group; F: The number of branching points in each group.

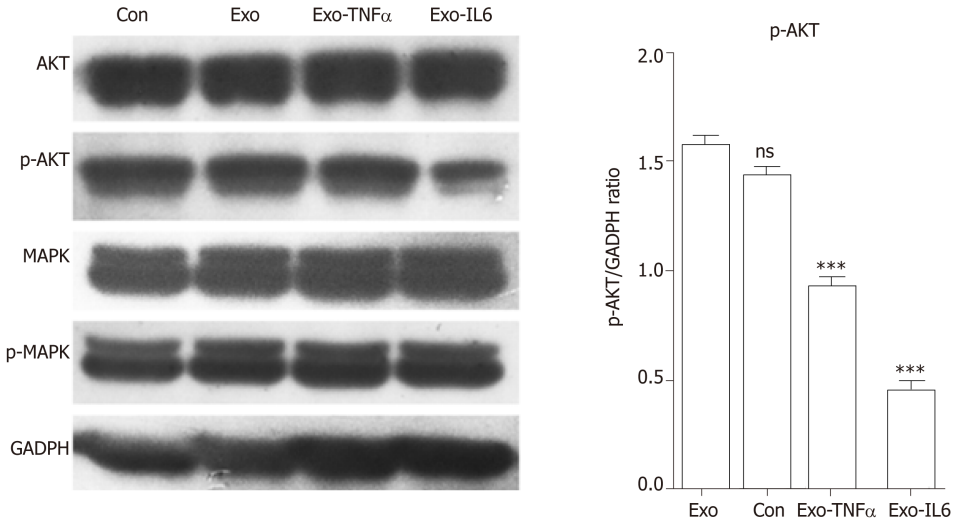


Figure 15 Western blot analysis. Western blot was performed to detect the expression of the indicated proteins in endothelial cells treated with phosphate buffered saline (control), mesenchymal stem cell exosomes (MSCs-exo), MSCs-exo$^{TNF\alpha}$ (stimulated with tumor necrosis factor α), MSCs-exo^{IL6} (stimulated with interleukin 6). GAPDH was used as an internal loading control. TNF: Tumor necrosis factor; IL6: Interleukin 6.

ARTICLE HIGHLIGHTS

Research background

Stem cell transplantation has been developing rapidly and has resulted in breakthroughs for the treatment of various diseases.

Research motivation

Treatments utilizing stems cells often require stem cells to be exposed to inflammatory environments, such as vascular cell adhesion molecule-1, tumor necrosis factor α (TNFα), and interleukin 6 (IL6). Stem cell-derived exosomes are especially important in producing miRNAs that impact angiogenesis.

Research objectives

MicroRNAs (miRNAs) are RNAs 0-20 nucleotides in length, which are derived from hairpin-like precursor miRNAs. They acts as important regulators of mRNA expression. It has been reported that miRNAs play critical roles in some cells and have the potential as diagnostic and therapeutic biomarkers.

Research methods

The morphology and quantity of mesenchymal stem cell (MSC) exosomes (MSCs-exo) are influenced by different inflammatory cytokine environments.

Research results

The morphology and quantity of each group of MSC exosomes were observed and measured. The miRNAs in MSCs-exo were sequenced. Differential expression of miRNAs and their target genes as well as the related regulatory mechanisms were researched.

Research conclusions

TNFα and IL6 may influence the expression of miRNAs that down-regulate the PI3K-AKT, MAPK, and VEGF signaling pathways; particularly, IL6 significantly down-regulates the PI3K-AKT signaling pathway.

Research perspectives

Overall, inflammatory cytokines may lead to changes in exosomal miRNAs that abnormally impact cellular components, molecular function, and biological process, particularly angiogenesis.

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META-ANALYSIS

Stem cell treatment and cerebral palsy: Systemic review and meta-analysis

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Abstract

BACKGROUND

Perinatal complications may result in life-long morbidities, among which cerebral palsy (CP) is the most severe motor disability. Once developed, CP is a non-progressive disease with a prevalence of 1-2 per 1000 live births in developed countries. It demands an extensive and multidisciplinary care. Therefore, it is a challenge for our health system and a burden for patients and their families. Recently, stem cell therapy emerged as a promising treatment option and raised hope in patients and their families.

AIM

The aim is to evaluate the efficacy and safety of stem cell treatment in children with CP using a systematic review and meta-analysis

METHODS

We performed a systematic literature search on PubMed and EMBASE to find randomized controlled clinical trials (RCT) investigating the effect of stem cell transplantation in children with CP. After the review, we performed a random-effects meta-analysis focusing on the change in gross motor function, which was quantified using the gross motor function measure. We calculated the pooled standardized mean differences of the 6- and/or 12-mo-outcome by the method of Cohen. We quantified the heterogeneity using the I-squared measure.

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RESULTS

We identified a total of 8 RCT for a qualitative review. From the initially selected trials, 5 met the criteria and were included in the meta-analysis. Patients' population ranged from 0.5 up to 35 years (n = 282). We detected a significant improvement in the gross motor function with a pooled standard mean difference of 0.95 (95% confidence interval: 0.13-1.76) favoring the stem cell group and a high heterogeneity (I^2 = 90.1%). Serious adverse events were rare and equally distributed among both intervention and control groups.

CONCLUSION

Stem cell therapy for CP compared with symptomatic standard care only, shows a significant positive effect on the gross motor function, although the magnitude of the improvement is limited. Short-term safety is present and further high-quality RCTs are needed.

Key words: Cerebral palsy; Perinatal brain injury; Stem cells; Umbilical cord blood; Mesenchymal stem/stromal cells; Gross motor function; Meta-analysis

Core tip: Cerebral palsy is a severe motor disability resulting from perinatal complications. Recently, stem cell therapy emerged as a promising treatment option and raised hope in patients and their families. However, high-quality randomized clinical trials investigating the efficacy of stem cell therapy as a treatment for cerebral palsy are scarce. We included the small number of currently available trials in our meta-analysis. A slight but significantly positive effect on the gross motor function favoring the stem cell group can be seen.

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INTRODUCTION

Despite advances in perinatal medicine, many infants continue to face serious risks during pregnancy, parturition, and adaptation after birth. Preterm birth and severe birth asphyxia are the most frequent complications, which may lead to brain damage of the newborn. The clinical presentation in an individual child after perinatal complications is complex. This complexity results from multiple potential causal pathways, signs and symptoms of injury. Typical pathology in these newborns include brain injury and the resulting cerebral palsy (CP). Therefore, CP summarizes a heterogeneous group of non-progressive disabilities in motor function which range from slight motor impairment of an isolated body part to the inability of walking or speaking[1]. Depending on the severity, typical symptoms are difficulties in coordination, stereotypic movement, impossibility of discrete movements, evocation of primitive reflexes through voluntary movements or co-contraction of agonist and antagonist resulting in spasticity. Multiple other impairments such as seizure disorders, altered sensation or perception or musculoskeletal disorders can appear as additional symptoms[1]. Although the diagnosis of CP does not require cognitive disability, about two-thirds of children with CP are confronted with it[2]. Not surprisingly, CP is the most common motor disability in children with a prevalence of 2 per 1000 live births in developed countries. The prevalence of CP increases exponentially in preterm infants with decreasing gestational age[3]. For example, the risk to develop CP is 30 times higher in infants born before 33 wk gestation compared to term-born infants[3]. Besides prematurity, major risk factors include placental abnormalities, major and minor birth defects, low birthweight, meconium aspiration, emergency caesarean section, birth asphyxia, neonatal seizures, respiratory distress syndrome, hypoglycemia, and neonatal infections[4]. Together, CP originates from a multifactorial pathology with multiple risk factors and in many cases, a distinct cause

is unclear.

Due to major improvements in neonatal care, half of the children suffering from CP are preterm infants and the other half are term-born infants[5,6]. This differentiation is important as prophylactic/therapeutic approaches differ in these two populations. Currently, the only intervention known to reduce the burden of CP in the term population is hypothermia. Hypothermia is associated with a significant reduction in death and disability in children subjected to perinatal asphyxia[7]. However, 40%-50% of infants treated with hypothermia still die or develop significant neurological disability[8]. In the preterm population, hypothermia is contra-productive and therapeutic options are lacking. One option is the antenatal magnesium sulfate prophylaxis at less than 30 wk of gestation, which reduces CP and combined CP and mortality rate at 2-years of age. However, long-term neurological benefits are lacking[9,10]. New avenues to treat CP emerged and stem cell treatments are particularly promising. We review the potential of a stem cell transplant using a meta-analysis to evaluate gross motor function after randomized controlled trials (RCTs) in children with CP.

Which source of stem cells to use?

Stem cells are characterized as cells with the capacity of self-renewal and differentiation into multiple tissues[11]. Their program of division and differentiation is regulated by the immediate microenvironment, also called the niche. Stem cells are grouped depending on the number of tissues they can differentiate into[12]. Totipotent cells can differentiate into any cell type found in an organism. They exist for a very limited time only in the embryo shortly after fertilization. Pluripotent cells are the next stage; they occur in the blastocyst and can form cells from each of the three germinal sheets. Multipotent stem cells can also be detected in adults. Their differentiation capability is restricted to cell types within one germinal sheet. Unipotent stem cells are responsible for the renewal of a single tissue lineage. *In vitro*, apart from their self-renewal and differentiation capacity, stem cells can be identified by markers, which are gene products expressed by specific types of stem cells. Therefore, by means of flow cytometry, stem cells can be identified and quantified[11].

Embryonic or pluripotent stem cells seem to be very promising due to their differentiation ability[11]. However, their unlimited self-renewal and differentiation capacity combined with a lack of cell-cell-interaction and regulation through extraembryonic cells may lead to tumor formation[13]. Additionally, the harvesting of pluripotent cells results in the death of the embryo raising major ethical concerns. Therefore, the main stem cell sources in CP treatment are bone marrow (BM) or umbilical cord blood (UCB)-derived stem cells.

Umbilical cord blood and bone marrow as a source of stem cells

UCB is a rich source of stem cells. It can be collected after birth and stored in public or private banks for a possible future use[14]. It contains several types of stem and progenitor cells, among which hematopoietic stem cells (HSC), mesenchymal stem/stromal cells (MSC) and endothelial progenitor cells (EPC) are the most relevant. Further, the existence of embryonic-like stem cells is controversially discussed. Besides the simple isolation without ethical concerns, these cells have a remarkably low immunogenicity. Compared to stem cells from other sources, UCB cells tolerate more human leukocyte antigen (HLA)-mismatches without rejection[12]. The reason for this might be the immature fetal immune system. Today, cord blood is routinely used to treat hematopoietic or immunologic disorders[15]. In the last years, promising trials have shown that UCB stem cells have great potential in the treatment of various neurological diseases[16]. A world-wide network of cord blood banks is available for unrelated cord blood transplantations. For therapeutic use, the mononuclear fraction is isolated from cord blood by means of a density gradient[17]. The mononuclear fraction includes immunosuppressive cells such as regulatory T-cells and monocyte-derived suppressor cells[16]. Each of these cell types has characteristics that are likely to contribute to neuroprotection. Notably, the composition of those cells depends on the timing of sampling (gestational age of pregnancy)[18]. For example, UCB derived from preterm placentae is different in its mononuclear fraction from term-derived, and UCB from intrauterine growth restriction infants has impaired EPC[19].

BM is the major hematopoietic organ localized in the central cavities of axial and long bones[20]. Differentiation and proliferation of the blood cells occur in the hematopoietic compartment. It is composed of HSC and the precursors for the different blood lineages. It used to be the main source of HSC for clinical use, until recently less invasive techniques allowed to obtain HSC from other sources such as granulocyte-colony stimulating factor-stimulated peripheral blood or UCB[21]. Importantly, the stroma is responsible for the regulation of the hematopoietic process

and contains MSC and EPC along with their products of differentiation like fibroblasts and endothelial cells. Compared to UCB, MSC are more frequent in BM. Although BM harvesting requires an invasive procedure, BM is still the main source for MSC[22]. Notably, the number of both EPC and MSC in BM decline with age[20,23].

Mesenchymal stem/stromal cells

MSC are a heterogeneous population of multipotent cells that can differentiate into bone (osteoblasts), fat (adipocytes), cartilage (chondroblasts) and periosteum (fibroblasts)[24]. Their differentiation potential into the neurogenic direction is debated, but the discussion remains controversial[17]. Originally, MSCs were discovered in BM as part of the mononuclear cell fraction, where they support HSC[25]. Meanwhile, they have been isolated from many other sources including adipose tissue, muscle, placental tissues, and UCB. The International Society for Cell and Gene Therapy elaborated minimal criteria to define MSCs[26]. It remains unclear and much discussed what MSC's function in each source is. Some even consider the pericyte to be the cell of origin of MSC *in vivo*. Thus, their function could be the regulation of the capillary blood flow and permeability[27]. There are high expectations for MSC as a therapy in various diseases. A special interest lies in the treatment of neurological diseases including CP[24]. Also, MSC have become more and more commercialized as source of replacement for damaged structures. However, injected allogeneic MSC are rejected by the host immune system and likely to be eliminated soon after the transplantation and in contrast, autologous ones may persist for some longer time[27]. Not surprisingly, the therapeutic effect of MSC is attributed not to the differentiation capacity and thus formation of new tissue, but to the secretome, which contains modulatory factors[28]. These modulate oxidative stress and has angiogenetic, anti-apoptotic and anti-inflammatory effects[28]. MSC have different characteristics depending on their origin. Of special interest are those MSC derived from fetal tissues such as UCB, placental or cord tissues, which are believed to have a wider differentiation and greater proliferation potential[29,30]. Moreover, they can easily be isolated non-invasively, rapidly and without ethical concern nor invasive procedures[31]. However, we are not always successful to isolate a sufficient number of MSC from UCB for clinical use[32].

Hematopoietic stem cells and endothelial progenitor cells

Both HSC and EPC can be found in BM as well as in UCB. HSC are multipotent precursor cells with the ability to form all blood cells while maintaining a sufficient HSC-pool to provide hematopoiesis throughout life[33]. Whilst HSC transplantation was the first established stem cell transplantation and exclusively for hematologic disorders, HSC now also become interesting in the field of non-hematologic diseases[34]. Of special interest for a possible neuroprotective contribution is their beneficial effect in ischemic brain injury in animal models[35]. EPC too are believed to possess neuroprotective features. Apart from their ability to differentiate into endothelial cells, they are believed to induce neovascularization and reduce hypoxia-induced apoptosis and destruction of blood vessels[36,37]. Considering these in-vitro characteristics of both HSC and EPC, they may play a role in neuroprotective therapies such as the treatment of CP as well.

MATERIALS AND METHODS

Literature search

We followed the PRISMA-statement guidelines to perform a systematic electronic search using PubMed and EMBASE databases for trials published between January 1, 1990, and February 11, 2019. To be eligible for inclusion, the trial was required to be a randomized, controlled clinical trial with full-text availability in English. Further, the study population must consist of children diagnosed with any type of CP. The intervention must include any kind of stem cell treatment compared to placebo and/or standard of care such as rehabilitation. The motor outcome of both intervention and control group must be assessed and reported in the Gross Motor Function Measure (GMFM). We excluded trials that did not meet these criteria from the analysis.

We combined terms like "stem cells", "neural progenitor cells", "mesenchymal stem cells" or "umbilical cord blood" to match the intervention with "cerebral palsy" in both databases. We set the limitations to human-controlled clinical trials in English only. Also, we detected trials from bibliographies of other articles. We identified the potentially relevant articles through title and abstract screening and proceeded to the definite inclusion or exclusion according to their content (supplementary materials). The study selection process is illustrated in Figure 1.

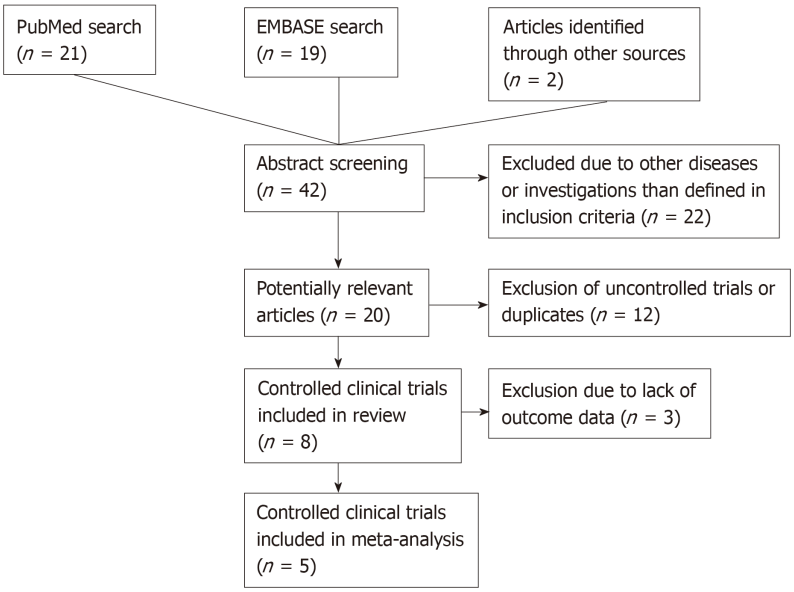

Figure 1 Study selection process.

Meta-analysis

We defined the efficacy of stem cell therapy as a change in gross motor function. To summarize the gross motor outcome from the intervention group compared to the control group, we performed a random-effects meta-analysis using the method of DerSimonian *et al*[38]. We pooled the standardized mean differences and the heterogeneity was quantified using the I-squared measure, taken from the inverse-variance fixed-effect model. All analyses were done in Stata version 15 using the command meta version 3.04.

RESULTS

Study selection and characteristics

After applying the inclusion and exclusion criteria, we identified 8 relevant trials for further analysis (Figure 1). Four of them have been conducted in China, three in Korea and one in the United States of America. Publication dates ranged from 2012 to 2018. The study characteristics are summarized in Table 1. Five trials investigated stem cell therapy plus rehabilitation to rehabilitation only, one trial compared stem cell therapy. only to rehabilitation and two trials compared stem cell therapy only to no intervention in the control group. Two of the included studies were three-group randomized clinical trials investigating the additive effect of erythropoietin[39] or using mononuclear cells[40]. Two other studies were designed as randomized controlled crossover-trials[41,42]. The age in the study population ranged from 6 mo to 35 years. All patients included were diagnosed with CP. The severity of CP was measured with the Gross Motor Function Classification System, which divides the syndrome of CP into 5 levels, whereof level 5 is the most severe motor impairment[43]. The type and dose of the stem cells differed in the trials. One trial used neural progenitor cells derived from aborted fetuses' forebrains[44], while others included umbilical cord- or BM-derived stem cells[39,41,45-47]. The applied dose ranged from 4×10^6 to 6×10^8 cells. The number of cells was not always adapted to body weight. The application route was in most cases the intravenous route. In summary, both the study population and the intervention characteristics are heterogeneous, which leads to a certain risk of bias summarized in Table 2 and Figure 2.

Risk of bias

We assessed the risk of bias in individual studies using the Cochrane criteria. The risk ranged from low to high. The major source of risk of bias was patient and personnel blinding[40,44-46]. The number of trials was not sufficient to estimate the risk for publication bias.

Outcome measurements

All included trials applied standardized scales to assess the neurodevelopmental

Table 1 Intervention and study population overview

		Study Population			Intervention					
Ref.	Country	Sample size	Age (yr)	Patient condition	Intervention and study groups	Application route	Type of stem cells	Source	Origin	Dose
Chen *et al*[45], 2013	China	60	1-35	CP, GMFCS level 3 to 5	Group 1: SC + rehabilitation Group 2: Rehabilitation only	Intrathecal	Mesenchymal stem cells *in vitro* transformed to neural stem cell-like cells	Bone marrow	Autologous	Fixed quantity of 1-2 × 10^7 cells, twice
Huang *et al*[46], 2018	China	56	3-12	CP	Group 1: SC + rehabilitation Group 2: Placebo + rehabilitation	Intravenous	Mesenchymal stem cells	Cord blood	Allogeneic	Fixed quantity of 5 × 10^7 cells, 4 times
Kang *et al*[47], 2015	South Korea	36	0.5-20	CP, GMFCS level 1 to 5	Group 1: SC Group 2: Placebo No rehabilitation	Intravenous/intra-arterial	Mononuclear cells	Cord blood	Allogeneic[1]	5.46 × 10^7 cells/kg, once
Liu *et al*[40], 2017	China	105	0.5-12.5	Spastic CP, GMFCS level 2 to 5	Group 1: MNC Group 2: MSC Group 3: rehabilitation	Intrathecal	Mesenchymal stromal cells and mononuclear cells	Bone marrow	Autologous	10^6 cells/kg, 4 times
Luan *et al*[44], 2012	China	91	< 0.5-3.5	CP, GMFSC level 1 to 5	Group 1: SC + rehabilitation Group 2: rehabilitation only	Intraventricular	Neural progenitor cells	Aborted healthy fetuses' forebrains	Allogeneic	Fixed quantity of 8-10 × 10^6 cells, once
Min *et al*[39], 2013	South Korea	105	0.8-10	CP	Group 1: SC+ EPO + rehabilitation Group 2: placebo SC + EPO + rehabilitation Group 3: placebo SC+ placebo + rehabilitation	Intravenous	Mononuclear cells	Cord blood	Allogeneic[1]	≥ 3 × 10^7 cells/kg, once
Rah *et al*[42], 2017	South Korea	57	2-10	Non-severe CP	Group 1: G-CSF at baseline, SC 1 mo later Group 2: G-CSF at baseline, placebo 1 mo later crossover at 7 mo	Intravenous	Mobilized mononuclear cells	Peripheral blood	Autologous	5.97 × 10^8 cells/ kg, once

Sun *et al*[41], 2017	USA	63	1-6	CP of any type, GMFCS Level 2 to 4 or 1 with hemiplegia	Group 1: SC + rehabilitation Group 2: Placebo + rehabilitation	Intravenous	Mononuclear cells	Cord blood	Autologous	2×10^7 cells/kg, once

[1]Administation of cyclosporine. MNC: Mononuclear cells; MSC: Mesenchymal stem cells; EPO: Erythropoietin; CP: Cerebral palsy; SC: Stem cells; GMFCS: Gross motor function classification system.

outcome. The fine and gross motor function, cognitive function and spasticity were measured. In the following analysis, we focused on the gross motor function outcome only. The gross motor function was evaluated with the GMFM-66 or GMFM-88[43]. Both scores categorize 5 sections: Lying, Sitting, Crawling, Standing and Walking. Each of these categories is expressed in the percentage of the maximum achievable points. To determine the total score, the percentages of all categories are added up and divided by five and therefore expresses the average score of all categories. Importantly, the total score varies depending on the severity of CP and the patient's age. The observation period in the included trials was 6 months in three trials, 12 mo in another three and 24 in the remaining two trials (see Table 3 for details). Functional assessments to measure the outcomes were performed at baseline, after the observation period, and additionally at one to three points in-between. Most GMFM data were available for the 6- and 12-mo periods and therefore we decided to proceed with those for the meta-analysis.

Cell dose, gross motor function and safety

Three trials from the initial literature search had to be excluded from the meta-analysis due to the lack of GMFM scores in an averaged format[45], missing standard deviation or standard error in the GMFM scale[42], or no comparable outcome score of the control group given[44]. One study did not provide changes in GMFM summary scores from baseline, but only absolute GMFM dimension scores at baseline, 6 months and 12 mo[40]. Therefore, we averaged the dimension scores and their variations and calculated the changes from the baseline. The cell dose used in the included trials differed but improved motor outcome seems to correlate with a higher cell dose[41,42,47] (Tables 1 and 3). We generated a forest plot with the available GMFM-scores at 6 and 12 mo (see Figures 3-5). Additionally, we pooled all studies; the resulting forest plot is presented in Figure 5. Besides the improvement of gross motor function, the studies assessed the serious adverse events (SAE). Overall, only two trials reported the occurrence of SAEs[39,44] (Table 4). One case of hemorrhagic foci after transplantation was detected and was presumably linked to the invasive procedure[44]. The other SAEs mainly consisted of infections or seizures[39]. These SAEs were equally distributed between both cell recipient and control groups. There is no report of an overdose in any of the included trials. The highest dose applied was a mean dose of 5.97×10^8 cells/kg and no SAEs were reported[42]. Together, the included RCTs provide short-term safety, whereas the long-term impact is unclear.

DISCUSSION

Using a systematic literature review, we identified 8 RCTs investigating the effect of stem cell treatment in children with CP *vs* standard care with or without placebo. Based on our inclusion criteria, we included 5 trials in the meta-analysis with a total of 282 patients (142 control, 140 cell recipient group). By combining the outcome, we identified a significant improvement in the cell recipient compared to the control group (Figure 5). The pooled standard mean difference was 0.95 (95% confidence interval: 0.13-1.76). However, we detected a high heterogeneity (I^2 = 90.1%), which reflects the diverse patient characteristics and needs to be accounted for while discussing the results. Besides, our analysis only includes the results from 5 trials, which is hardly enough to represent the whole CP population. A search on clinicaltrials.gov identified 9 ongoing randomized clinical trials about stem cell therapy in CP. Therefore, evidence might grow stronger in the future with greater data acquisition.

In the included trials, the origin (autologous or allogeneic), type (MSC, mononuclear cells, and neural progenitor cells), and source (BM, brain tissue, UCB) of the transplanted cells differed. Interestingly, in patients receiving the transplant with

Table 2 Evaluation of the risk of bias

Ref.	Random sequence generation: Selection bias	Allocation concealment	Blinding of participants and personnel: Performance bias	Blinding of outcome assessment: Detection bias	Incomplete outcome data: Attrition bias	Selective reporting
Chen *et al*[45], 2013	High	High	High	Low	High	High
Huang *et al*[46], 2018	Unclear	Unclear	High	Low	Low	Unclear
Kang *et al*[47], 2015	Low	Low	Low	Low	Low	Unclear
Liu *et al*[40], 2017	Low	Unclear	High	Unclear	Low	Low
Luan *et al*[44], 2012	Unclear	Unclear	High	Unclear	High	Unclear
Min *et al*[39], 2013	Low	Low	Low	Low	Low	Low
Rah *et al*[42], 2017	Low	Low	Low	Low	High	Unclear
Sun *et al*[41], 2017	Low	Low	Low	Low	Low	Unclear

fewer HLA-mismatches, the improvement of gross motor function was clearly evident[39,47]. We speculate that autologous cells may show a greater benefit in motor function improvement. Furthermore, the matching of HLA seems to have less relevance in case of a MSC transplant[46]. For example, both studies with a significant increase in the motor outcome used a MSC transplant[40,46]. These observations are further supported by the direct comparison of mononuclear fraction of BM stem cells and MSC alone[40], which clearly favors a MSC alone approach. Together, the present studies suggest that MSC are the ideal candidates to modulate the gross motor function in CP. The source may be the UCB as it is less invasive and easily accessible. However, harvesting a sufficient number of cells can only be achieved in around 40% of the samples[48], which limits the use of autologous UCB-derived MSC. Further studies are warranted especially as other easily available MSC sources such as Wharton`s jelly of the umbilical cord are very promising[49,50].

In order to report a proper gross motor function improvement, both the patient's age and level of motor impairment need to be considered by using percentiles[51]. However, only one of the included trials used proper percentiles and presented the results as "greater than expected"[41]. Interestingly, this trial detected a significant gross motor function improvement using a higher cell dose (≥ 2×10^7 cells) only. Another consideration is the use of repeated cell applications[40,46]. Given that the pathophysiology of CP consists of persistent inflammation, such repetitive doses would be plausible[52]. Moreover, the transplanted cells do not persist for a very long time[53], which further supports concurrent use. Eventually, the included trials do not clearly provide a specific effective dose of cells, but it should be more than 2×10^7 total nucleated cells and may go up to 6×10^8 without the risk of overdosing[41,42]. The invasive transplantation routes such as the lumbar puncture were chosen to bypass the blood-brain barrier but resulted in a higher number of reactions like nausea and headache[40]. However, the modulation of inflammatory responses by secretion of factors can be achieved peripherally[54], making the route of application less relevant for the efficacy of the cells[55].

In conclusion, stem cell therapy for CP compared with symptomatic standard care only, shows a significant positive effect on the gross motor function, although the magnitude of the improvement is limited. Especially MSC show a positive outcome. This novel treatment seems safe, at least in the short term. However, high-quality RCTs are still lacking.

Table 3 Raw motor outcome data

Ref.	Follow-up assessments after intervention (mo)	Change in GMFM-88/66 after 6 mo ± SD	Change in GMFM-88/66 after 12 mo ± SD	% improvement compared to baseline after whole observation period
Chen *et al*[45], 2013	1, 3, 6	Numbers inaccurately indicated	-	-
Huang *et al*[46], 2018	3, 6, 12, 24	Control: + 2.96 ± 1.66; MSC: + 7.62 ± 2.44	Control: + 4.75 ± 1.45; MSC: + 10.27 ± 2.96	Control: 5.67%; UCB: 14.89%
Kang *et al*[47], 2015	1, 3, 6	Control: 3.85 ± 3.75; UCB: 7.08 ± 7.36		Control: 13.1%; UCB: 24.2%
Liu *et al*[40], 2017	3,6, 12	Control: + 1.85; BMMNC: + 3.1; BMMSC: + 10.45	Control: + 2.91; BMMNC: + 6.46; BMMSC: + 12.52	Control: 7.84%; BMMSC: 33.76%
Luan *et al*[44], 2012	1, 6, 12	*Control: not indicated;* NPC: + 8.86	-	-
Min *et al*[39], 2013	1, 3, 6	Control: + 7.8 ± 5.1; EPO only: + 9.0 ± 6,3; UCB + EPO: + 9.1 ± 6.7	-	No baseline score provided
Rah *et al*[42], 2017	12 mo after treatment resp. 6 mo for crossover-group	-	Transplantation at baseline: + 2.9; Transplantation after 6 mo: + 6.37	No baseline score provided
Sun *et al*[41], 2017	12, 24	-	Control: + 6.9 ± 5.5; UCB: + 7.5 ± 6.8 (High dose: improvement + 4.3 ± 1.5 greater than expected. Low-dose and placebo: no significant improvement beyond expectation.)	Control: 13.3%; UCB: 15.3%

BMMNC: Bone marrow mononuclear cells; BMMSC: Bone marrow mesenchymal stem cells; EPO: Erythropoietin; NPC: Neural progenitor cells; UCB: Umbilical cord blood; MSC: Mesenchymal stem cells; GMFM: Gross Motor Function Measure.

Table 4 Reported serious adverse events

Ref.	Serious adverse events in cell recipient group	Serious adverse events in control group	Allogenic cells	Application route
Chen *et al*[45], 2013	0/30	0/30		Intrathecal
Huang *et al*[46], 2018	0/27	0/27	x	Intravenous
Kang *et al*[47], 2015	0/18	0/18	x (+ immunosuppressant)	Intravenous/intraarterial
Liu *et al*[40], 2017	0/68	0/34		Intrathecal
Luan *et al*[44], 2012	1/45	0/49	x	Intraventricular
Min *et al*[39], 2013	3/36	6/70	x (+ immunosuppressant)	Intravenous
Rah *et al*[42], 2017	0/57	-		Intravenous
Sun *et al*[41], 2017	0/63	-		Intravenous
Total	4/344 (1.2%)	6/228 (2.6%)		

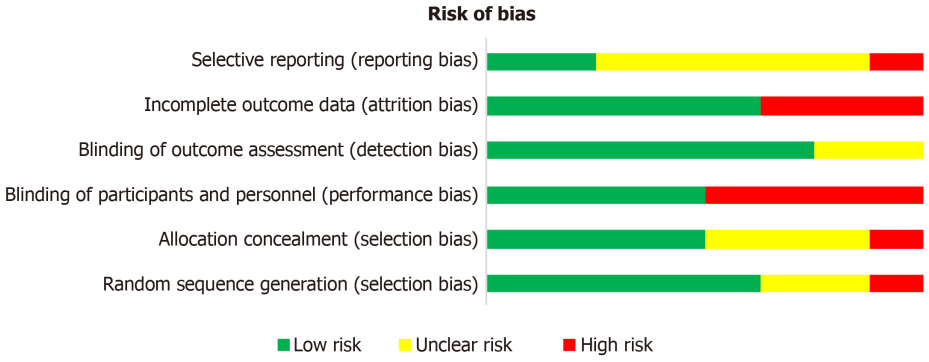

Figure 2 Overall risk of bias.

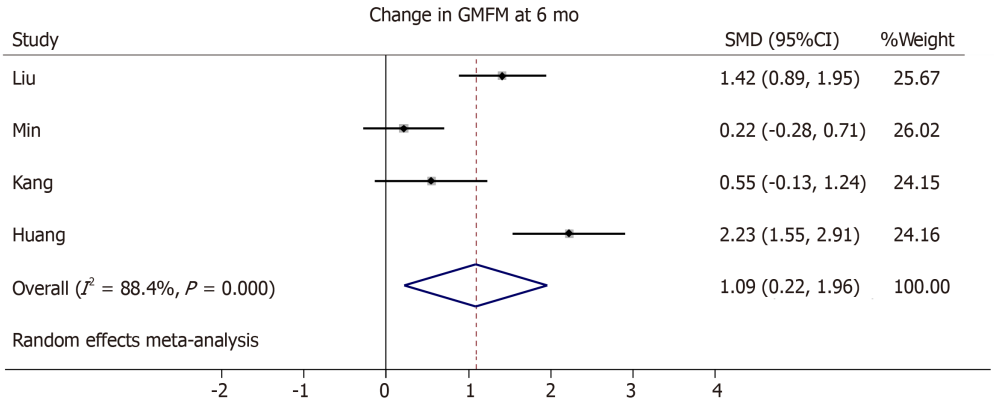

Figure 3 Forest plot showing the effect size of the change in the Gross Motor Function Measure in the intervention group compared to the control group after 6 mo. GMFM: Gross Motor Function Measure; SMD: Standard mean difference; CI: Confidence interval.

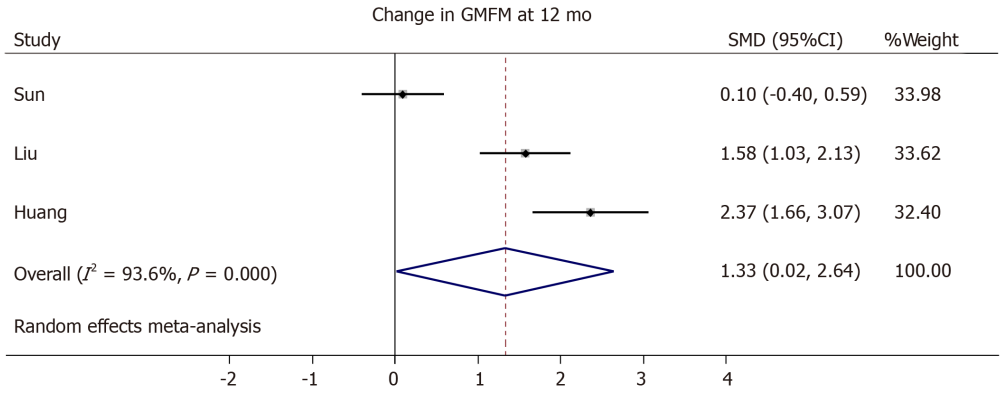

Figure 4 Forest plot showing the effect size of the change in the Gross Motor Function Measure in the intervention group compared to the control group after 12 mo. GMFM: Gross Motor Function Measure; SMD: Standard mean difference; CI: Confidence interval.

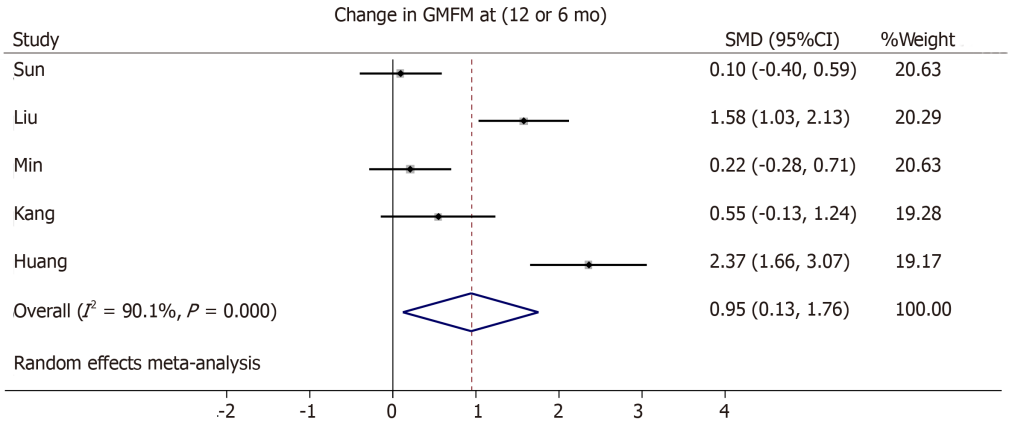

Figure 5 Forest plot showing the effect size of the change in the Gross Motor Function Measure in the intervention group compared to the control group. If available, measures at 12 mo were used[40,41,46], else, measures at 6 mo were used[39,47]. GMFM: Gross Motor Function Measure; SMD: Standard mean difference; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Cerebral palsy (CP) is a severe, uncurable motor disability resulting from perinatal complications. It is a challenge for our health system and a burden for both patients and their families.

Research motivation
During the last years, stem cell therapy emerged as a novel treatment option. Clinical trials report promising results and create high expectations. However, most trials are of poor quality and neither randomized nor controlled, so the scientific evidence remains doubtful.

Research objectives
The aim of our meta-analysis was to investigate the effect of stem cell treatment on the gross motor function in children with CP.

Research methods
With a systematic literature search on PubMed and EMBASE, we identified the eligible randomized controlled clinical trials (RCTs). We performed a random-effects meta-analysis focusing on the change in gross motor function and calculated the pooled standardized mean differences of the 6- and/or 12-mo-outcome.

Research results
We identified a total of 8 RCTs for a qualitative review. From the initially selected trials, 5 met the criteria and were included in the meta-analysis. Patients' population ranged from 0.5 up to 35 years (n = 282). We detected a significant improvement in the gross motor function with a pooled standard mean difference of 0.95 (95% confidence interval: 0.13-1.76) favoring the stem cell group and a high heterogeneity (I^2 = 90.1%). Serious adverse events were rare and equally distributed among both intervention and control groups.

Research conclusions
Stem cell therapy for CP compared with symptomatic standard care only, shows a significant positive effect on the gross motor function, although the magnitude of the improvement is limited.

Research perspectives
Considering that this small number may not be enough to represent the whole CP population, our meta-analysis detected a small but significant improvement in the gross motor function favoring the stem cell group. However, the magnitude of the effect is limited. In the future, high-quality research with a more homogenous study population is needed to bring more clarity.

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