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Innovations in Cancer Research and Regenerative Medicine

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Tissue Engineering and Regenerative Medicine

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Tissue Engineering and Regenerative Medicine

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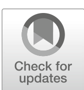

Physico-Mechanical Properties of HA/TCP Pellets and Their Three-Dimensional Biological Evaluation In Vitro

Nurulain 'Atikah Kamalaldin, Mariatti Jaafar, Saiful Irwan Zubairi, and Badrul Hisham Yahaya

Abstract

The use of bioceramics, especially the combination of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), as a three-dimensional scaffold in bone engineering is essential because together these elements constitute 60% of the bone content. Different ratios of HA and β-TCP were previously tested for their ability to produce suitable bioceramic scaffolds, which must be able to withstand high mechanical load. In this study, two ratios of HA/TCP (20:80 and 70:30) were used to create pellets, which then were evaluated in vitro to identify any adverse effects of using the material in bone grafting. Diametral tensile strength (DTS) and density testing was conducted to assess the mechanical strength and porosity of the pellets. The pellets then were tested for their toxicity to normal human fibroblast cells. In the toxicity assay, cells were incubated with the pellets for 3 days. At the end of the experiment, cell morphological changes were assessed, and the absorbance was read using PrestoBlue Cell Viability Reagent™. An inversely proportional relationship between DTS and porosity percentage was detected. Fibroblasts showed normal cell morphology in both treatments, which suggests that the HA/TCP pellets were not toxic. In the osteoblast cell attachment assay, cells were able to attach to the surface of both ratios, but cells were also able to penetrate inside the scaffold of the 70:30 pellets. This finding suggests that the 70:30 ratio had better osteoconduction properties than the 20:80 ratio.

Keywords

Bioceramics · HA/TCP · 3D scaffold · Bone substitution · Cell evaluation · Cell toxicity

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Abbreviation

HA	Hydroxyapatite
β-TCP	β-Tricalcium phosphate
3D	Three-dimensional
TCP	Tricalcium phosphate
αMEM	Alpha Minimum Essential Medium
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
AA	Antibiotic-antimycotic
IMR-90	Normal human fibroblast
OSTB	Osteoblast
ATCC	American Type Culture Collection
DTS	Diametral tensile strength
MPa	Megapascal
N	Newtons
SEM	Scanning electron microscopy
FESEM-EDX	Field-emission scanning electron microscopy with energy dispersive X-ray spectroscopy

1 Introduction

The use of a scaffold for bone and dental tissue engineering applications is important for osteo-integration, osteoinduction, and osteoconduction (Bosco et al. 2012; Ghanaati et al. 2012; Laschke et al. 2010; Tzaphlidou 2008). The scaffold should stimulate and enhance the maturation of stem cells for tissue regeneration and provide a suitable surface for cellular attachment and homing (Castilho et al. 2014; Dhaliwal 2012; Javaid and Kaartinen 2013; Marolt et al. 2010). Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are common bioceramic materials that are used in bone and dental tissue engineering applications (Bagher et al. 2012; Jang et al. 2012). The chemical structures of these compounds are similar to those of the mineral components of bone and teeth, which makes them suitable for addition to the three-dimensional (3D) scaffold used for bone and dental grafting (Detsch et al. 2008; Di Filippo et al. 2006; Duan et al. 2005).

Despite numerous reports of the bioconductivity of HA (Detsch et al. 2008; Di Filippo et al. 2006; Miao et al. 2007) and the bioactivity of β-TCP as bone graft materials (Bagher et al. 2012), application of these materials has some limitations. For example, HA has poor mechanical properties, is brittle and rigid, has low wear resistance, and exhibits slow biodegradability. In contrast, β-TCP has a faster biodegradation rate (Hutmacher and Garcia 2005; Macchetta et al. 2009; Nookuar et al. 2011; Rezwan et al. 2006) and high mechanical strength (Miao et al. 2008; Panzavolta et al. 2009; Tas et al. 2007). When designing a scaffold for bone applications, the chosen materials or composites should have high mechanical strength and exhibit favorable conditions for osteoblast and mesenchymal stem cell homing (Panzavolta et al. 2009; Zhang et al. 2005). Previous studies have shown that combining HA and β-TCP in different ratios results in more stable 3D scaffolds (Ramay and Zhang 2004; Rezwan et al. 2006; Roldán et al. 2007; Sulaiman et al. 2014; Zhang et al. 2005) and better cellular behavior during the engraftment and healing processes (Bagher et al. 2012; Ghanaati et al. 2012; Jang et al. 2012).

Ghanaati et al. (2012) used a combination of HA and β-TCP at a 60:40 ratio (in weight percentage, wt%) and reported that it was able to induce rapid vascularization and integration during the early stage of implantation in 3D bone scaffolds, providing a good setting for slow degradation, which is crucial for bone tissue regeneration (Ghanaati et al. 2012). The fast degradation rate of TCP-alone scaffolds may lead to failure in clinical implantation due to inadequate osteoconductive properties. Detsch et al. (2008) found only a few multinucleated osteoclast cells (precursor cells for osteoblasts) on the TCP surface, whereas more were found on the HA surface (Detsch et al. 2008), likely because HA has a slower degradation rate compared to TCP.

The combination of HA and β-TCP ceramics can increase the strength of the scaffold for high load and mechanical applications (Kivrak and Tas 1998; Laschke et al. 2010; Miao et al. 2008). For example, the HA/β-TCP composite

was able to provide a platform for adapting the inflammatory response during healing and degradation processes with optimal new boney structure ingrowth (Ghanaati et al. 2012). A composite's ability to attract cells for homing is critical for facilitating successful scaffold grafting. Using a 20:80 ratio of HA/TCP in electrospun composite, Oryan et al. (2014) demonstrated that human mesenchymal stem cells can differentiate into the osteogenic lineage with a high proliferation rate and a high production of boney matrix in vivo (Oryan et al. 2014).

Finding the optimal ratio of HA and β-TCP with excellent mechanical properties and porosities with no toxicity to cells and tissues is crucial for bone grafting applications. In this study, two ratios of HA and β-TCP (20:80 and 70:30) were used to create pellets to produce 3D bone scaffolds. Their abilities to induce cellular homing, attachment, and cellular differentiation were assessed, and their mechanical strength and toxicity were measured. The biocompatibility of HA/TCP to serve as a surface for cellular attachment was also evaluated.

2 Materials and Methods

2.1 Materials

Materials used in this experiment were HA, β-TCP powder, and glutaraldehyde (Sigma-Aldrich). Alpha Minimum Essential Medium (αMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS), and antibiotic-antimycotic (AA) were obtained from GIBCO. PrestoBlue Cell Viability Reagent™ was purchased from Invitrogen, and normal human fibroblasts (IMR-90) and osteoblasts (OSTB) were obtained from the American Type Culture Collection (ATCC).

2.2 Pellet Fabrication

The pellets weighed 1 g each and contained 20:80 (w/w) or 70:30 (w/w) ratios of HA and β-TCP. The HA and β-TCP powder were added and stirred for 1 h to ensure that the combinations were homogenized. A stainless steel cylindrical mold (4 cm × 1.3 cm) was used (mold area, 5.2 cm^2), and the HA/TCP pellets were produced by applying 20 psi pressure for 10 s. The pellets then were sintered at 1250 °C for 60 min to produce dense pellets.

2.3 Diametral Tensile Strength (DTS) Testing

Cylindrical pellets with diameter to length ratio of 1:2 were used for DTS testing. The testing was performed using an Instron 4505 with 10 kN load with crosshead speed set at 1.0 mm/min. The load was gradually applied until the pellets broke in half. Five samples of each ratio were tested ($n = 5$). The DTS value (Della 2008; Klammert et al. 2009) was calculated using Eq. (1):

$$DTS\ (\delta t), MPa = 2F/\Pi dh \tag{1}$$

where:

F = fracture load in Newtons (N)
D = diameter of specimens
H = height of specimens
Π = pi value; 3.1416

2.4 Density Testing

The Archimedes method was used to determine the porosity of the pellets by first measuring their density. Five samples from each ratio were tested for porosity. To determine the density, the volume and bulk density were calculated using Eqs. (2) and (3):

$$\text{Volume, V}\ \left(\text{cm}^3\right) = W_w(\text{g}) - W_s(\text{g}) \tag{2}$$

$$\text{Bulk density}, p\ \left(\text{g/cm}^3\right) = W_D(\text{g})/V\ \left(\text{cm}^3\right) \tag{3}$$

The dry weight (W_D) of the pellets was determined first. The pellets then were immersed in 250 ml of distilled water and placed in a desiccator for 2 h to ensure water absorption throughout the porous structures. The pellets then were weighed to obtain the immersed weight (W_s) [weight of the pellets during immersion] and wet weight (W_w) [weight of the pellets right after immersion]. Using the density, the porosity percentage was calculated using Eq. (4):

$$\text{Percentage of porosity}, P\% = (W_w - W_D)/V \times 100 \quad (4)$$

2.5 Pellet Sterilization

Prior to in vitro testing, the HA/TCP pellets were immersed in 70% ethanol (*v*/*v*) for 2 h and then rinsed three times with 1x PBS to completely remove the traces of ethanol in the pellets. The pellets were air-dried in a sterile environment and then sterilized with ultraviolet light for another 2 h. This step was to ensure the removal of any possible contaminants on the surface of the pellets. The sterilization method in this study was adapted and modified from Ryan J (Ryan 2005).

2.6 Evaluation of Cell Toxicity

The toxicity of the HA/TCP pellets to normal cells was tested using a cell viability assay. In this assay, the toxicity of the materials (pellets) toward cells tested was defined by the reduction in cell growth to below 50% viability. Three different groups of experiments were set up for the study: (1) control group (cells cultured with complete growth medium only), (2) cells cultured with 20:80 HA/TCP pellets, and (3) cells cultured with 70:30 HA/TCP pellets. IMR-90 cells were maintained in complete αMEM containing 10% FBS and 1% AA in an incubator supplied with 5% CO_2 at 37 °C. Cells were maintained until confluence was reached and then seeded for toxicity assays at 1×10^4 cells per well in 24-well plates. Prior to testing, the sterile HA/TCP pellets were immersed in complete αMEM overnight. Twenty-four hours after cell seeding, old αMEM medium in each well was replaced with fresh αMEM. The HA/TCP pellets were then transferred into each well and left to incubate for 3 days in a CO_2 incubator. Morphological changes of the cells were observed using an inverted microscope (Olympus), which was followed by assessment of toxicity of the pellets. The toxicity assay was carried out at days 1, 2, and 3 using PrestoBlue Cell Viability Reagent™. This reagent is resazurin based (a membrane permeable solution that reduces to form resorufin, which is a red fluorescent compound that can be detected and measured to determine cell viability). In a viable cell environment, the metabolic product of the cells is released into the culture medium, which later reduces the resazurin to resorufin, resulting in a change from blue to a pinkish red color. However, in a nonviable environment, the blue color of the resazurin is maintained because reduction does not occur. The absorbance of the samples was done in triplicate using an automated ELISA reader. The cell toxicity percentage was calculated using Eq. (5):

$$\text{Cell toxicity}\% = (Abs_{\text{treatment}}/Abs_{\text{control}}) \times 100\% \quad (5)$$

2.7 Cell Attachment Assay

This assay was performed to evaluate the ability of the scaffold to serve as a surface that enhances cellular attachment and homing. A total of 1×10^4 OSTB cells was seeded on top of each pellet, followed by incubation in a CO_2 incubator for 21 days to allow cell attachment (Chang et al. 1999; Schwartz et al. 2003). At day 21, the pellets were collected and fixed with 10% glutaraldehyde prior to scanning electron microscopy (SEM) imaging of the pellet surface as well as the cross section.

2.8 Field-Emission Scanning Electron Microscopy with Energy-Dispersive X-Ray Spectroscopy (FESEM-EDX) Analysis for Surface of HA/TCP Pellets

Cellular attachment on the pellets was observed using SEM imaging (Carl Zeiss). Prior to SEM imaging, HA/TCP pellets from the cell attachment assay were fixed using an ethanol gradient and 10% glutaraldehyde. The pellets were air-dried completely before being coated with gold particles for imaging. Pellets were analyzed for surface elements using the EDX analyzer.

2.9 Statistical Analysis

All data in this study were expressed as the mean ± standard deviation of triplicate analysis. Statistical differences among the data were subjected to statistical analysis using SPSS 20. Differences at $p < 0.05$ were considered as significant.

3 Results

3.1 Pellet Structure and Morphology

In this study, HA and β-TCP were combined in two different ratios (20:80 and 70:30), which resulted in different surface areas (0.42 cm^2 and 0.44 cm^2, respectively) (Fig. 1a). The differences between both pellets might be due to the different compositions of HA and β-TCP used. Further observation on the surface structure showed that there was porous structure produced (Fig. 2), with compact structure observed by 70:30 ratio. The porous structure of the pellets was also influenced by the different ratios of HA and β-TCP, as the 20:80 ratio had a higher porosity (23.31 ± 4.28%) compared to the 70:30 ratio (14.23 ± 3.82%) (Fig. 1b; Fig. 2). In the DTS testing, the 20:80 pellets were only able to withstand a 589.07 ± 91.85 MPa load, whereas the 70:30 pellets were able to withstand a 1022.72 ± 79.11 MPa load (Fig. 2).

3.2 Cell Toxicity Assay

The cell viability percentage represents the population of the viable cells in a population after being tested with interest compound or particle, wherein in this study they were the 20:80 and 70:30 HA/TCP pellets. In this assay, the data showed that both ratios of HA/TCP were not toxic on fibroblast cell. The toxicity was defined by the decrement in the cell viability percentage below 50% of cell growth. From this data, there was increment on the cell viability percentage, wherein above 90% of growth for 20:80 ratio and above 100% of growth for 70:30 ratio (Fig. 3 (graph)). The finding was then supported by the morphological observation of the fibroblast cell. All tested population showed normal fibroblast morphology, which was spindle-shaped morphology, and the distribution of the cell growth in both pellets tested was similar to the control population (Fig. 3 (cell morphology)).

3.3 Osteoblast Proliferation and Attachment Assay

SEM imaging revealed osteoblast cells attached to the pellets' surface (Fig. 4). The morphological structure of the cells that attached to the surface of the 20:80 pellets' surface was irregular, suggesting that the cells were still adapting to the various porous structures of the pellet. In contrast, the cells attached to 70:30 pellets exhibited a smear-like morphology, suggesting that the cells had already penetrated inside the structure. This finding was supported by the imaging of the cross sections of the HA/TCP pellets. In the 20:80 pellets, OSTB cells were only found in the top area and were not present in the bottom area (Fig. 5), whereas in the 70:30 pellets the cells were present throughout the pellet (Fig. 6).

Fig. 1 (**a**) Top and side view of the 20:80 and 70:30 HA/TCP pellets fabricated using a cylindrical mold. The diameter and height values of each pellet are 1.2 cm and 0.35 cm for the 20:80 ratio and 1.1 cm and 0.40 cm for the 70:30 ratio. (**b**) The porous structure (P) of the 20:80 and 70:30 ratio pellets. Magnification: 8000x

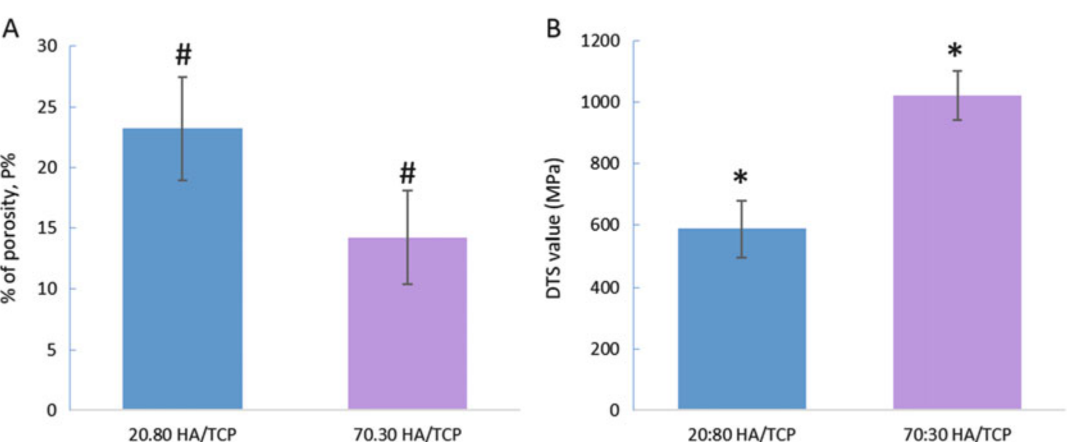


Fig. 2 Porosity percentage (**a**) and DTS (**b**) of the 20:80 and 70:30 HA/TCP pellets. The more porous structure (20:80 HA/TCP pellets) resulted in lower DTS, and the less porous structure (70:30 HA/TCP pellets) produced higher DTS values. P-value: * = 0.0037 and # = 0.0082

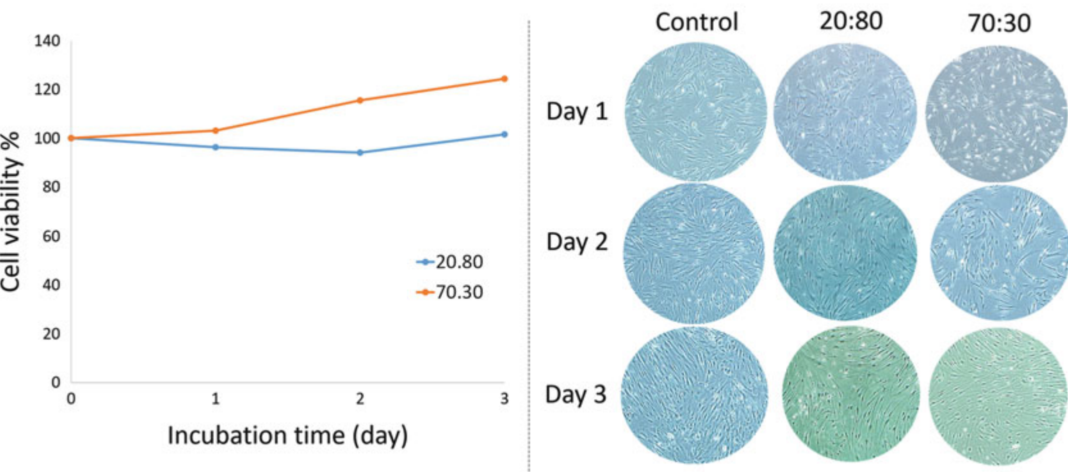


Fig. 3 The cell toxicity assay of both 20:80 and 70:30 HA/TCP pellets. *Graph*: Evaluation of cell viability and cell toxicity during exposure of IMR-90 cells to the 20:80 and 70:30 HA/TCP pellets for 3 days. No toxicity was detected following treatment with both ratios. *Cell morphology*: There were no morphological changes observed between control and both HA/TCP pellets. The cell exhibits normal morphology of the fibroblast which was spindle-shaped. *Magnification: x20*

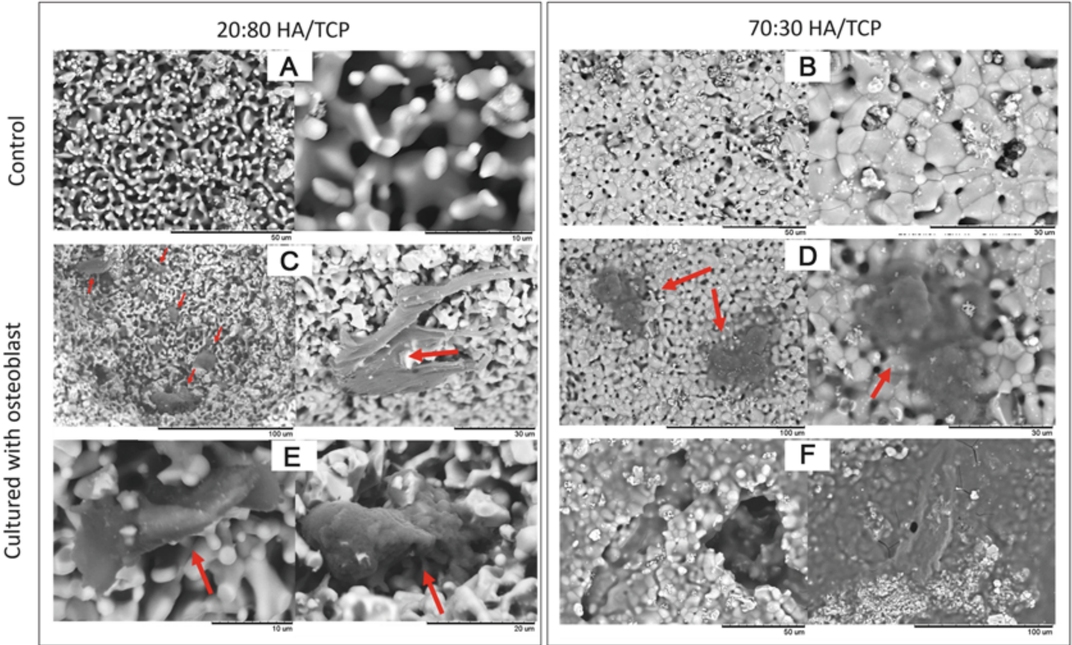


Fig. 4 SEM imaging of top view of 20:80 and 70:30 HA/TCP pellets (**a**, **b**). SEM images of the surface of 20:80 and 70:30 HA/TCP pellets, respectively (**c**, **d**, **e**, **f**). SEM images of the surface of the pellets after culture with osteoblast cells for 21 days. Cells are the dark bodies on the pellets, as indicated by red arrows. For the 20:80 HA/TCP pellets (**c**, **e**), osteoblast cells were starting to attach to the surface of the pellet, and the shape of the cell is seen clearly visible. For the 70:30 HA/TCP pellets (**d**, **f**), osteoblast cells had penetrated inside the scaffold, and the attached osteoblasts already formed a thin layer of film on the surface

Fig. 5 SEM imaging of a cross section of a 20:80 HA/TCP pellet. Osteoblast cells were distributed only on the top area of the 20:80 HA/TCP pellet (**a**, **b**, and **c**). No cells were observed on the bottom area (**d** and **e**). The pellet appeared as a white background, and the osteoblast cells were visible as dark bodies

Fig. 6 SEM imaging of a cross section of a 70:30 HA/TCP pellet. Osteoblast cells were observed throughout the pellet structure, on both the top and bottom areas (**a**–**e**). The pellet appeared as a white background, and the osteoblast cells were visible as dark bodies within the pellet structure

3.4 FESEM-EDX Analysis of the Pellets' Surface

Foreign particles with apatite-like crystal structure were observed on the surface of the 70:30 pellets (Fig. 7(a, b)); they were scattered on top of the osteoblast layer that attached to the pellets (OSTB layer). FESEM-EDX analysis was conducted to identify the possible elements in the particles, and the data showed increased levels of oxygen (O), sodium (Na), and calcium (Ca) and decreased levels of carbon (C) compared to the control pellets (without cell culture treatment).

However, our primary analysis of the ion profile of the apatite-like crystals revealed the presence of high concentrations of Na^+ and Cl^- ions, which might have originated from the traces of PBS used during the washing step (Fig. 8). Therefore, we reanalyzed these samples after rinsing the pellets ten times with distilled water instead of PBS. The reanalyzed data revealed that both Na^+ and Cl^- ions were removed (Fig. 9), thus eliminating the earlier assumption that the presence of the Na^+, K^+, Ca^{2+}, and Cl^- ions was due to the FBS and led to the formation of the bone-like apatite on the 70:30 pellets.

4 Discussion

HA and β-TCP were combined in two different ratios (20:80 and 70:30), which resulted in different surface areas (0.42 cm^2 and 0.44 cm^2, respectively). β-TCP, with a molecular weight of only 310.10 $gmol^{-1}$, tended to shrink during the sintering process compared to HA (502.31 $gmol^{-1}$). This trend is in accordance with previous work by Prakasam et al. (2015), who found that calcium phosphates (CaPs) undergo removal of gases and organic compounds followed by shrinking of the powder, which ultimately leads to the decrease in surface area (Prakasam et al. 2015). In designing the scaffold, surface area plays a crucial role because increased surface area increases the interaction between the material and the body tissue, thus enhancing the body's responses (De Jong 2008). According to Florence et al. (2006), the reactivity of the HA/TCP scaffold increases with increasing HA/TCP ratio: the higher the HA/β-TCP ratio, the better the reactivity in the bone remodeling process, thus enhancing the formation of apatite crystals from dissolution of the β-TCP implant during formation of new bone structure (Barrère et al. 2006). This process involves the supersaturation of Ca and P ions, which are required for spontaneous nucleation of the calcium crystal on the scaffold's surface (Salgado et al. 2004). The formation of apatite crystals within the biomaterial structure will promote the adsorption of proteins and formation of the protein layer, which will encourage adhesion of osteoblasts during new boney structure formation (Nookuar et al. 2011; Tas et al. 2007).

The porous structure of the pellets was influenced by the different ratios of HA and β-TCP, as the 20:80 ratio had a higher porosity (23.31 ± 4.28%) compared to the 70:30 ratio (14.23 ± 3.82%) (Fig. 1b; Fig. 2). In the DTS testing, the 20:80 pellets were only able to withstand a 589.07 ± 91.85 MPa load, whereas the 70:30 pellets were able to withstand a 1022.72 ± 79.11 MPa load (Fig. 2). Bone grafting requires a scaffold that can withstand tension resistance of 50–151 MPa for cortical bone and 1–5 MPa for spongy bone (Prakasam et al. 2015; Salgado et al. 2004). In fabricating a scaffold with high mechanical strength, higher porosity is a detriment. This is due to the nature of the 3D structure, in which high porosity is associated with low mechanical strength and vice versa. This theory was proven in this study. The porosity and DTS values exhibited an inversely proportional relationship (Prakasam et al. 2015), and the 70:30 ratio produced strength that was twice that of the 80:20 ratio. The optimal pore size for bone tissue engineering ranges from 200 to 900 μm (Salgado et al. 2004). Smaller pore size (< 200 μm) will cause inefficient nutrient flow and prevent neovascularization of new blood vessels and cellular penetration and migration (Cao and Kuboyama 2010; Dhaliwal 2012; Rezwan et al. 2006),

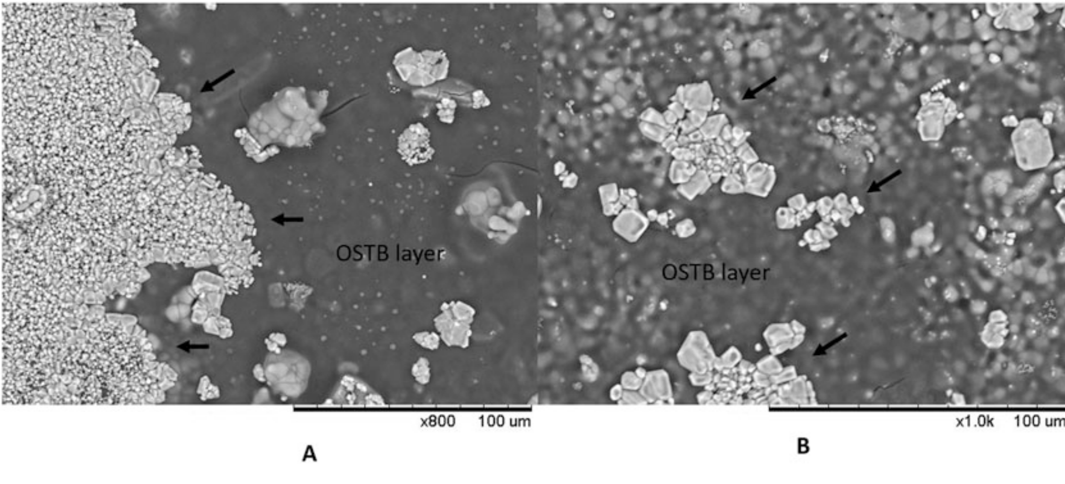
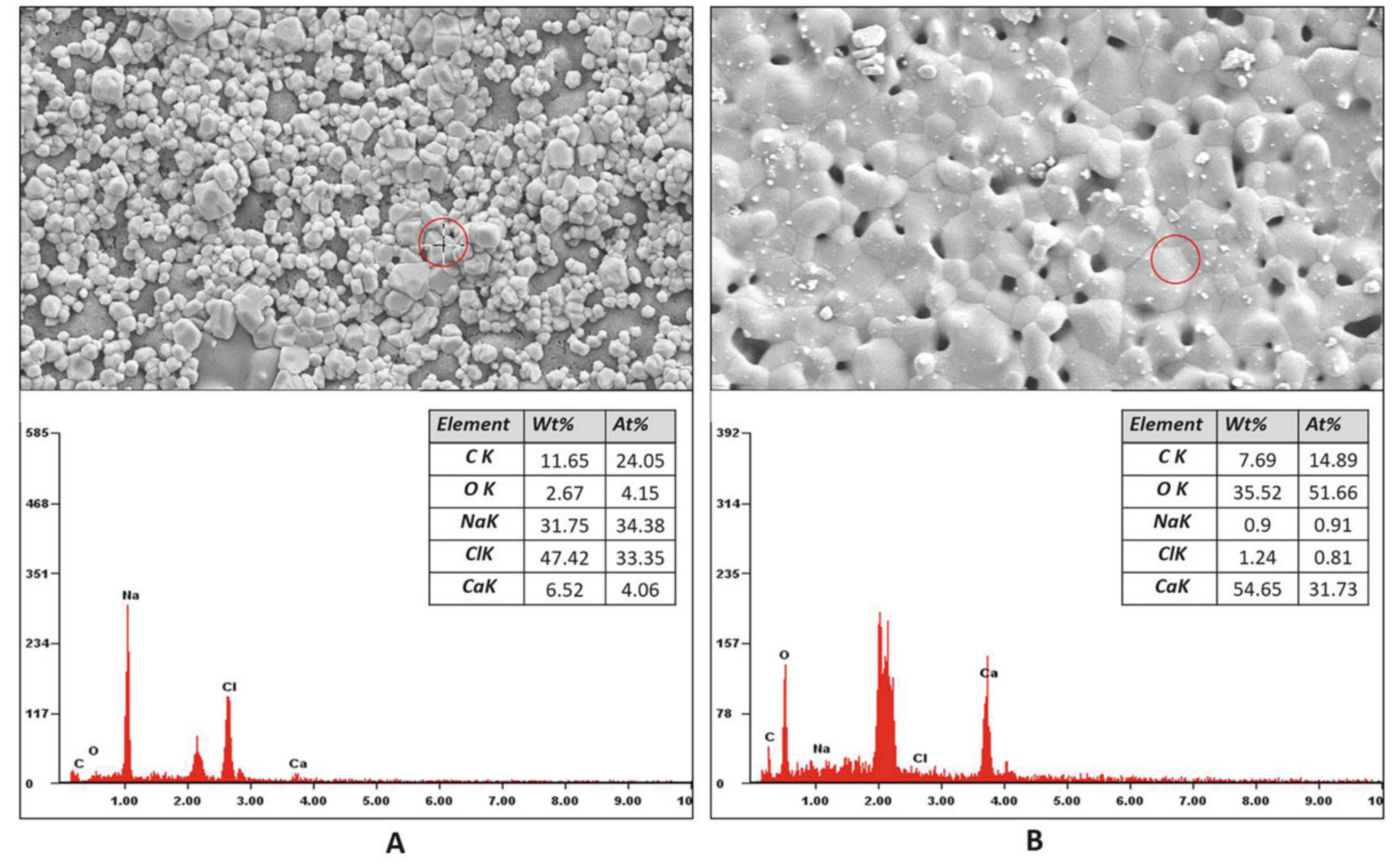


Fig. 7 (**a**) The appearance of the foreign particles on top of the osteoblast (OSTB) layer. The crystallized structure of the particles mimicked the apatite-like structure. (**b**) Higher magnification of the structure (1000x)



Element	Wt%	At%
C K	11.65	24.05
O K	2.67	4.15
NaK	31.75	34.38
ClK	47.42	33.35
CaK	6.52	4.06

Element	Wt%	At%
C K	7.69	14.89
O K	35.52	51.66
NaK	0.9	0.91
ClK	1.24	0.81
CaK	54.65	31.73

Fig. 8 FESEM-EDX analysis of (**a**) an osteoblast-treated pellet and (**b**) an untreated pellet. Foreign particles with apatite-like structure were observed on the surface of (**a**). Both figures represent the 70:30 HA/TCP pellets

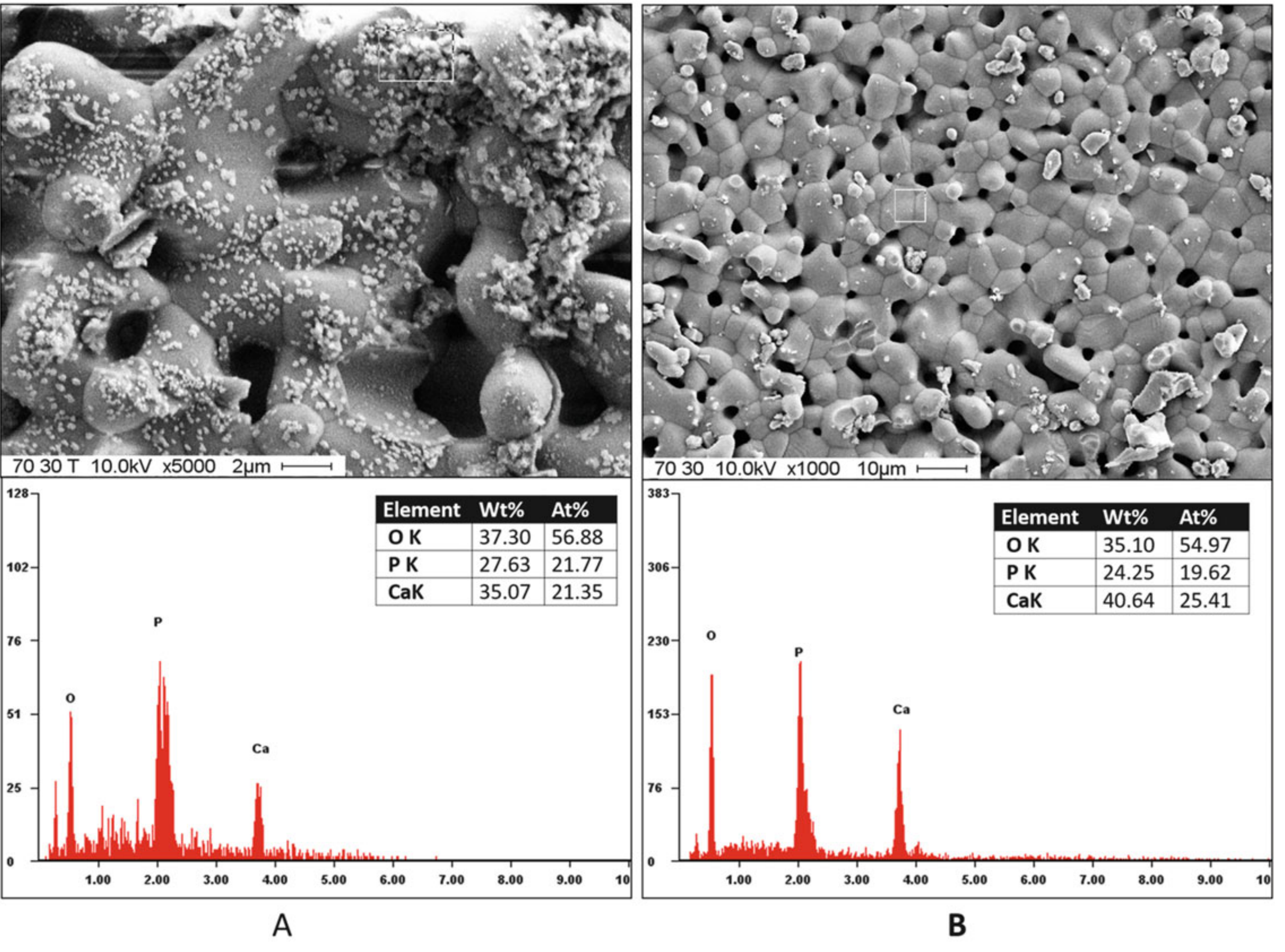


Fig. 9 FESEM-EDX analysis post-washing of the 70:30 HA/TCP pellets with distilled water: (**a**) osteoblast-treated pellet and (**b**) untreated pellet. The Na and Cl ions were undetected in (**a**)

whereas larger pores (1.2–2.0 mm) are better for allowing cellular penetration.

The ability of the HA/TCP pellets to serve as homing structure for osteoblast was observed through osteoblast proliferation and attachment assay. In this study, the differences in osteoblast distribution throughout the structure of the pellets might be due to the different HA/TCP ratios, porosity of the pellet, and availability of the surface area for cellular attachment and homing. Sulaiman et al. (2014) described the use of the 20:80 HA/TCP ratio as a potential candidate for bone scaffold for the formation of tissue-engineered bone in nude mice Miao et al. (2007). They reported that the ceramic composite was able to induce the formation of new boney structure at the surface of the ceramic, which later migrated to the center of the scaffold. Other studies reported that the HA/TCP scaffold is ideal for surface attachment of cells and extracellular matrix synthesis (Macchetta et al. 2009; Roldán et al. 2007). Several different ratios of HA and TCP have been tested in vivo, and the tested ratios (15:85, 35:65, 85:15, 60:40) were able to initiate the calcination of the compounds and led to osteoblast formation (Roldán et al. 2007; Zhang et al. 2005). To achieve an optimal surface for cell attachment, surface modifications can be made either by protein addition or plasma treatment (Rezwan et al. 2006).

αMEM complete medium formulation used in this study contains ion concentration of Na^{+}, 137.0; K^{+}, 11.0; Ca^{2+}, 136.0; and Cl^{-}, 103.0, which originated from the FBS. Mineral deposition on a scaffold can be induced by stimulated body fluid that contains ion concentrations (Na^{+}, 142.0; K^{+}, 5.0; Mg^{2+}, 1.5; Ca^{2+}, 2.5; Cl^{-}, 148.0; HCO_3^{-}, 4.2; HPO_4^{2-}, 1.0; SO_4^{2-}, 0.5 mM) that mimic those of human blood plasma (Duan et al. 2005; Kawashita et al. 2009; Monteiro et al. 2003). Thus, the presence of some of these ions in the αMEM complete medium (Na^{+}; K^{+}, Ca^{2+}, and Cl^{-}) might initiate mineralization of the bone-like apatite on the surface of the 70:30 HA/TCP pellets.

5 Conclusion

The data suggest that both HA/TCP ratios (20:80 and 70:30) have potential to be used as bone scaffolding in treating bone defects or facilitating bone growth and replacement following trauma. This finding was supported by the in vitro study in which no toxic properties were observed when tested on IMR-90 cells. Osteoblast attachment on both ratios and penetration of the 70:30 HA/TCP pellets were observed, suggesting that both ratios were able to serve as a surface for cellular attachment and homing. However, cell viability and cellular penetration inside the pellets differed between the 20:80 and 70:30 pellets, likely because of the different HA and β-TCP compositions. The 70:30 ratio served as a better platform for cellular ingrowth and penetration even though the porosity percentage was lower compared to that of the 20:80 ratio. Surface modification of 70:30 pellets will be conducted in the future, as it might be a way to initiate the formation of bone-like apatite.

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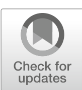

The Robust Potential of Mesenchymal Stem Cell-Loaded Constructs for Hard Tissue Regeneration After Cancer Removal

Samaneh Hosseini, Mohammad Amin Shamekhi, Shahrbanoo Jahangir, Fatemeh Bagheri, and Mohamadreza Baghaban Eslaminejad

Abstract

Malignant bone tumors, although quite rare, are one of the causes of death in children and adolescents. Surgery as a common and primary treatment for removal of virtually bone cancer cause large bone defects. Thus, restoration of hard tissues like bone and cartilage after surgical tumor resection needs efficient therapeutic approaches. Tissue engineering (TE) is a powerful approach which has provided hope for restoration, maintenance, or improvement of damaged tissues. This strategy generally supplies a three-dimensional scaffold as an active substrate to support cell recruitment, infiltration, and proliferation for neo-tissues. The scaffold mimics the natural extracellular matrix (ECM) of tissue which needs to be regenerated. The use of potent cell sources such as mesenchymal stem cells (MSCs) has also led to remarkable progresses in hard tissue regeneration. Combination of living cells and various biomaterials have continuously evolved over the past decades to improve the process of regeneration. This chapter describes various strategies used in TE and highlights recent advances in cell-loaded constructs. We herein focus on cell-based scaffold approach utilized in hard tissue engineering and parameters determining a clinically efficient outcome. Also, we attempt to identify the potential as well as shortcomings of pre-loaded scaffolds for future therapeutic applications.

Keywords

Mesenchymal stem cells · Hard tissue regeneration · Cancer Removal · Stem cell loaded constructs

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1 Introduction

Bone, as a dynamic tissue, provides a well-favored microenvironment for development of cancer cells. Indeed, bone cancer as one of the prevalent cancers is commonly caused by trauma, inflammation, infection, or abnormal tissue growth. It destroys the healthy bone and failure of early treatment leads to metastasis to the lungs and liver (Kansara et al. 2014). Bone cancer can be categorized into primary and secondary types based on where the cancer was started. In primary bone cancers which are either benign (e.g., osteoma, osteoid osteoma, osteoblastoma, osteochondroma, and enchondroma) or malignant (osteosarcoma, Ewing's sarcoma, and chondrosarcoma) tumors, bone is the first organ in which tumor is formed. The primary bone cancer comprises 0.2% of all human neoplasms among young adults and adolescents with low long-term survival rates (Hameed and Dorfman 2011). On the other hand, in secondary bone cancer, the tumor spreads from other organs to bone. Current clinical treatment of localized malignant tumors is the combination of various approaches including directed tumor resection, radiation, and systemic pre- and postoperative chemotherapy. Indeed, surgery is a priority in treatment to avoid amputation of patients' cancerous bone. Surgeons remove the tumor lesions along with some surrounding healthy tissue (to avoid the risk of growing new tumors) that leads to critical-sized defects. Therefore, reconstruction of large bone void is a surgical challenge that needs to be tackled to restore the bone function. Traditionally, the autologous bone grafts, synthetic prosthesis, or cadaveric allografts have been used as a substitute for the lost bone (Benevenia et al. 2016; Marulli et al. 2017). However, the complications induced by the traditional approaches have hampered their extensive clinical applications. Limited volume and donor site morbidity are autografts complications. Alternatively, allografts as an excellent bone graft are less commonly used due to the risk of immunogenicity, reduced bioactivity, and high cost of sample preparation, handling, and storage (Brydone et al. 2010; Oryan et al. 2014). Tissue engineering (TE), as a recent thriving research area, offers a remarkable possibility of replacement of the lost or damaged tissue with engineered structure (Langer and Vacanti 2016). The tissue-engineered products made from combination of cells, scaffolds, and bioactive molecules have expanded promising horizons in medicine. Tremendous efforts have been made to create novel structures with high regenerative capacity. To restore bone critical-sized defects observed following surgery such as limb salvage surgery, scaffold-based approaches could apparently be feasible and beneficial. Moreover, numerous attempts made in the recent years have also verified the substantial role of cells in bone healing (Khojasteh et al. 2008). Although the most favorable cell type required for tissue regeneration is cells originated from the same tissue as the tissue that needs to be regenerated (e.g., osteoblasts for bone, chondrocytes for cartilage, and tenocytes for tendon), a number of obstacles have hampered their use. Donor site morbidity, limited numbers of harvested cells, further degeneration during harvesting, and decreases in proliferation and dedifferentiation following in vitro expansion are the most common drawbacks. Discovery of various types of stem cells, including mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs), has made them potent cell sources for TE applications (Hosseini and Baghaban Eslaminejad 2017). Among various types of stem cells, MSCs are of crucial importance since they can be derived from almost all tissues and have the potential of differentiating into multiple cell lineages, such as chondrocytes and osteoblasts, under appropriate external stimuli (Allickson et al. 2011; Dehghan et al. 2015; Guilak et al. 2004; Jackson et al. 2010; Jiang et al. 2002; Jones et al. 2008; Lee et al. 2004, 2016; Patki et al. 2010; Witkowska-Zimny and Wrobel 2011). Encouraging results of MSC-based bone healing obtained in clinical trials have revolutionized the field of bone TE (Eslaminejad and Nadri 2009; Hosseini and Eslaminejad 2016; Mattioli-Belmonte et al. 2015; Oryan et al. 2017). Considering significant regenerative capacity of MSCs, combination of these cells

with various scaffolds would be an appealing restoration strategy. Here, we first describe various strategies employed in TE focusing on cell-loaded scaffolds for hard tissue regeneration. Due to chemical, physical, and mechanical interplays between cells and scaffolds, this chapter discusses the parameters that affect the clinical outcomes of cell-loaded constructs used for hard tissue repair. We also review the application of MSC-loaded constructs in clinical and preclinical studies in bone, cartilage, and tendon repair.

2 Various Strategies Employed in TE for Hard Tissue Replacement

The field of tissue engineering encompasses various approaches based on the application of its fundamental elements such as cells, scaffolds, and bioactive agents to develop a biological substitute with normal function and structure. Cell-free, bioactive-loaded, and cell-loaded scaffolds comprise three main categories in TE (Fig. 1). Below, these categories are described in details.

2.1 Cell-Free Scaffolds Strategy

Distinct scaffold properties such as porosity, permeability and interconnectivity support attachment, migration, proliferation, and distribution of host cells affect vascular bone regeneration and eventually encourage the ingrowth of surrounding bone. This phenomenon known as osteoconductivity is fundamental to scaffold-based strategy in TE. As an example, scaffolds made of hydroxyapatite (HA) possess sufficient

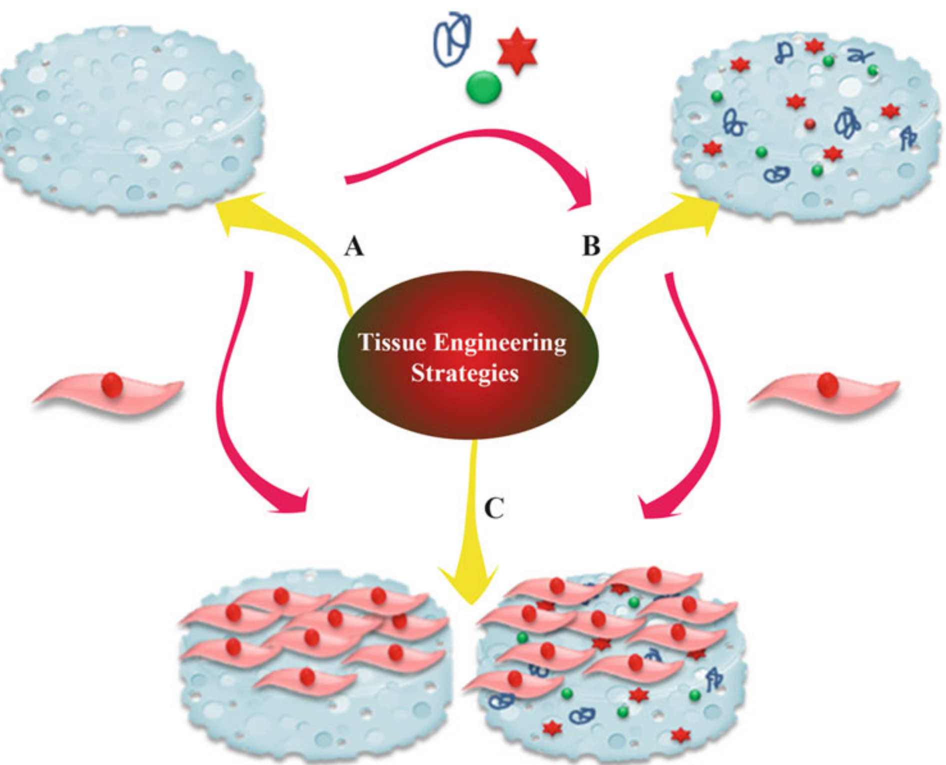

Fig. 1 Three common approaches utilized in TE: (**a**) cell-free scaffolds, (**b**) bioactive-loaded scaffolds, and (**c**) cell-loaded scaffolds

osteoconductivity since new bone formation occurred around the scaffold (Scaglione et al. 2012). In this strategy, the scaffolds are not pre-seeded with cells. The effectiveness of bone formation in the regeneration process is generally dependent on various factors such as microstructure, porosity, surface chemistry, and in vivo biodegradation rate of the scaffold (Woodard et al. 2007; Zhao et al. 2017) (see Sect. 3). New blood vessels are formed along with migration of soft tissues into the scaffolds and fibro vascular tissue formation. Bone forming cells are conducted to the defect site via neo-formed blood vessels, which facilitate the healing process. To achieve efficient osteogenesis and integration to the host tissue, scaffolds need to mimic the bone structure, morphology, and function. For example, in a study, calcium phosphate scaffolds were fabricated to mimic the natural structure of long bones. The constructed scaffolds simultaneously met the requirements of inner macroporous structure for in vivo degradation and those of outer nano-porous structure for mechanical integration (Lindner et al. 2014). Several synthetic biomaterials such as HA and its polymer nanocomposites have been extensively studied as alternative osteoconductive materials to bone. The mineral features of these materials mimic the natural structure of bone (Fernandez et al. 2011).

2.2 Bioactive-Loaded Scaffolds Strategy

Scaffolds are assumed to be intrinsically able to stimulate migration and differentiation of stem cells in adjacent host tissue. However, almost all synthetic materials need bioactive or signaling molecules to acquire this capability. Thus, activation of scaffolds using signaling molecules is a common strategy to induce skeletal development (Blackwood et al. 2012). Platelet-derived growth factors (PDGFs), epidermal growth factor (EGF), transforming growth factor beta (TGF-β) superfamily, fibroblast growth factor (FGF), and parathyroid hormone (PTH) are some cytokines that have been widely used in hard tissue regeneration studies (Bone and Process 2017; Buket Basmanav et al. 2008; Kang et al. 2015; Son et al. 2015; Vo et al. 2012). Bone morphogenetic proteins (BMPs) are the most studied osteoinductive agents (Fan et al. 2015; Nie et al. 2017). BMPs are involved in a vast majority of cellular processes like proliferation and differentiation. They regulate MSCs differentiation during bone and cartilage formation and maintain bone homeostasis by acting on a tetrameric receptor complex (Chen et al. 2012; Wu et al. 2016).

Due to some limitations in the use of growth factors such as short half-lives and high cost, small molecules were introduced as an alternative to facilitate bone healing process (Balmayor 2015; Faghihi et al. 2013). Small molecules are organic molecules that are generally smaller than 1000 Da, and their hydrophobic structure enables them to pass through the cell membrane and trigger signaling pathways (Balmayor 2015). Our group reported that fibrous PCL scaffolds containing dexamethasone, as an osteoinductive molecule, are able to induce osteogenic activity of MSCs (Omidvar et al. 2016). Recently, Wang et al. has discussed a number of small molecules that have stimulatory effect on osteogenic and chondrogenic properties of MSCs (Wang et al. 2015).

2.3 Cell-Based Scaffold Strategy

The process of tissue formation and mineralization using scaffolds pre-seeded with one or more cell types is considered as an efficient strategy in TE. Ultimate goal of this approach is maintenance of cells such as MSCs, osteoblasts, and chondrocytes together with deposited extracellular matrix during tissue formation. The constructive role of MSCs and scaffolds and their interplay provide a potent platform for neo-tissue formation and vascularization. Along with promotion of vascularization inside the scaffold, other cells in the neighborhood migrate toward the defect site and join these residing cells, which in turn result in successful integration to adjacent tissue at the defect site. This strategy has been extensively conducted in

in vitro and in vivo studies to investigate parameters that may affect the result (Eslaminejad et al. 2007; Jafarian et al. 2008; Zandi et al. 2010). These parameters including the cell type, cell source, and scaffold material and structure are comprehensively discussed below.

3 Requirements of Hard Tissue-Engineered Scaffolds

Scaffold regulates and directs growth of the cells that are either migrating from adjacent tissues into the scaffold (in vivo) or those pre-seeded onto the scaffold (in vitro or in vivo). The scaffold serves as a mechanical support for the cellular behavior such as proliferation and differentiation (Bose et al. 2012).

So far, numerous scaffolds made from various materials have been used to regenerate different tissues and organs in the body. Concerning materials used in hard tissue engineering, bioceramics as bioactive organic materials (e.g., hydroxyapatite (HA), bioactive glasses, and calcium phosphates) have been regarded as the most promising biomaterials due to their characteristics such as high mechanical stiffness (Young's modulus), low elasticity, and hard brittle surface (Canillas et al. 2017). They have the ability to strongly bond to bone and form bone-like hydroxyapatite layers (Gao et al. 2014; Gerhardt and Boccaccini 2010; Hench 1998). On the other hand, the major drawback of ceramic scaffolds is the difficulty in controlling their degradation rate (O'Brien 2011).

Scaffolds fabricated from synthetic polymers including polystyrene, poly-l-lactic acid (PLLA), polyglycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA), poly(urethanes), and poly(propylene fumarate) provide uniform microstructure, proper degradation rate, and mechanical strength, yet with reduced bioactivity and increased risk of rejection (Ozdil and Aydin 2014). Natural biomaterials are the third class of materials used for preparation of scaffolds for tissue regeneration. The advantage of natural polymer scaffolds is having a high degree of scaffold-tissue compatibility due to their known chemical structure in biological recognition processes. However, obtaining homogeneous and reproducible structures is still challenging. Poor mechanical properties are another problem of scaffolds made of natural polymers, which limit their use in hard tissue and load-bearing orthopedic applications (Mano et al. 2007).

Encouraging results have been achieved following the use of scaffolds fabricated from proteins (e.g., collagen, albumin, and fibrin) and polysaccharide-based natural materials (e.g., hyaluronan, alginate, agarose, and chitosan). For example, collagen is considered as an ideal material since it is the major fibrous protein in the extracellular matrix (ECM). It offers strength and structural integrity to connective tissues like skin, bone, tendons, cartilages, blood vessels, and ligaments (Alaribe et al. 2016; Zohora and Azim 2014). Silk fibroin scaffolds also comprise another protein-based material category, and high strength bone tissue-engineered construct based on silk fibroin has also been fabricated (Mandal et al. 2012; Ribeiro et al. 2015).

Hydrogels are the most appropriate candidates for cartilage tissue engineering. Cartilage is a strong tissue, not as hard as bone that has the ability to withstand compression and retention due to its high liquid content (up to 80% of wet weight of articular cartilage). It is structurally composed of two solid and fluid phases. Hydrogels are made up of polymer chains, which swell in an aqueous solution and mimic cartilage structure. They are typically biocompatible, owing to their large water content, as they do not provoke immune responses and inflammation. Fibrinogen, collagen, alginate, gelatin, chitosan, and hyaluronic acid are natural biomaterials used for fabrication of hydrogel scaffolds. Synthetic biomaterials used in hydrogels are PLA, PEG, and its derivatives. Hydrogels can also be used in an injectable form to repair irregularly shaped defects and for cosmetic surgery (Alaribe et al. 2016).

Commonly, scaffolds are fabricated artificially or by decellularization of natural tissues. Fabrication of scaffolds is highly dependent on the applied biomaterial. For example, most ceramic scaffolds are prepared by sintering process. Solvent casting and particulate leaching,

gas foaming, phase separation, and freeze drying are other methods used for scaffold preparation. Other alternative techniques such as electrospinning and melt molding/particulate leaching as well as modern methods such as 3D printing (3DP), selective laser sintering (SLS), and fusion deposition modeling (FDM) have also been investigated for the production of scaffolds (Roseti et al. 2017; Thavornyutikarn et al. 2014).

Decellularized ECM (dECM) scaffolds prepared by decellularization methods support cell adhesion, proliferation, and differentiation to regenerate tissue. Using perfusion system and soaking tissue in mild detergent solution are also used as two common methods for tissue decellularization. The former use native vascular network to perfuse the solution, while the latter is mainly used for tissues that do not have easy access to their vasculature. Lack of immunogenicity, due to removal of cellular and antigenic components, makes dECM scaffolds suitable for implantation. Additionally, they preserve the ECM network and components including collagen and elastin fibers, proteoglycans, and vascular network of the native tissue which provide mechanical strength, elasticity, and hydration for load-bearing applications (Chan and Leong 2008; Fu et al. 2014; Garreta et al. 2017; Seetapun and Ross 2017). As an example, porcine articular cartilage was decellularized by chemical reagents, and DNA content was reduced up to 90%, while a little reduction in collage contents and structure occurred. This construct possesses the ability to support viability, proliferation, and chondrogenic differentiation of fat pad-derived MSCs (Luo et al. 2016). A number of studies have been performed to achieve naturally derived tendon scaffold by decellularization (Lovati et al. 2016). Youngstrom et al. used a combination of mechanical, detergent, and enzymatic protocols to remove cell components from equine tendon. They succeeded in preserving native 3D architecture without any significant alteration in biomechanical properties following decellularization (Youngstrom et al. 2013). Likewise, Ning et al. reported that their decellularized tendon slices (DTSs) kept native proteoglycans (fibromodulin and biglycan) and growth factors (TGF-b1, IGF-1, VEGF, and CTGF) up to 93%, and the tensile strength was 85.62% of that of native tendon (Ning et al. 2012).

Apart from materials and fabrication methods, there are several common features such as biocompatibility and biodegradability that should be taken into consideration when designing a scaffold to be used in any tissue type. Mechanical properties and scaffold architecture are two specific features for hard tissue engineering applications. Additionally, appropriate pore size, porosity, interconnectivity, and permeability of scaffold facilitate the transport of nutrient, waste, and extracellular matrix deposition (Bose et al. 2012). In the following sections, crucial properties of the scaffolds are discussed.

3.1 Biocompatibility

Biocompatibility has crucial importance in immediate integration of scaffold-host tissue. Biocompatibility of a material is defined as having no cytotoxicity, exhibiting low or no immunogenicity, and lacking carcinogenic effects. Biocompatible materials must not degrade into acidic residues that are toxic for cells. Several studies showed the inflammatory responses upon implantation of incompatible materials (Ehashi et al. 2014). Among various materials, natural materials exhibit high degree of biocompatibility for scaffold fabrication. For example, decellularized human bone and bovine bone are two biocompatible scaffolds that have been used for bone tissue engineering applications (Kakabadze et al. 2017; Shahabipour et al. 2013).

3.2 Biodegradability

Biodegradation is considered as a mandatory feature of implanted scaffolds in order to eliminate the need for additional surgery for removal of material (like metallic implants) from the body. Degradation rate should match that of the neo-tissue formation, as mechanical strength is mainly governed by the scaffold specifically in

early stages of bone healing. Indeed, by the time the defected tissue is completely renewed, the scaffold must fully degrade. If the rate of scaffold degradation exceeds that of the neo-bone formation, the scaffold fails to provide appropriate mechanical properties (Sultana 2013).

3.3 Porosity, Pore Size, and Level of Pore Interconnectivity

Bone tissue needs to be highly vascularized to support the cells with nutrients and remove the metabolites from the injured site. Indeed, vascularization facilitates the transfer of macrophages, neutrophils, osteoblasts, mesenchymal stem cells, and bioactive molecules to the site of injury leading to the callus formation. Also, it should be noted that higher porosity and pore interconnectivity results in better vascularization. Moreover, high porosity improves mechanical interlocking between the scaffold and the bone region of the host tissue (Hannink and Arts 2011). Porosity may also affect cell adhesion, thanks to high surface-to-volume ratio which improves cell-scaffold interactions. Pore interconnectivity has an optimum value as lower pore interconnectivity leads to poor blood vessel formation and higher one may affect cell seeding efficacy and cellular adhesion (Bai et al. 2010). Generally, the minimum porosity required for scaffolds is about 90%, because obtaining high interconnectivities with porosities of lower than 90% is so difficult. It should be mentioned that mechanical properties of the scaffolds are inversely proportional to the porosity values. Regarding the importance of mechanical properties, considerable attempts have been made to optimize the aforementioned parameters. Pore size values ranging between 50 and 710 μm have been proposed for bone tissue engineering applications.

A number of in vitro and in vivo experiments using various cell types (e.g., MSCs and osteoblasts) have been conducted to assess the effect of porosity on osteogenesis (Griffon et al. 2005). Increasing the porosity of collagen-hydroxyapatite composites from 49 to 79% did not increase the proliferation rate of MC3T3-E1 pre-osteoblasts cells (Itoh et al. 2004). In contrast, proliferation of rat BMSCs seeded onto non-woven fibers of polyethylene terephthalate (PET) scaffolds, increased by higher porosity from 93 to 97% which is related to enhancement of oxygen and nutrient delivery. However, the maximum expression of ALP and osteocalcin, as markers of osteogenesis, was obtained at lower porosities (~93%) following culture in normal and osteogenic medium for 4 weeks. Cell aggregation at lower porosities resulted in higher expression of these markers (Takahashi and Tabata 2004).

As mentioned earlier, cells and other bioactive agents diffuse into pores of the scaffold from adjacent tissues inside the body. Therefore, higher values of porosity improve the osteogenesis. Aarvold et al. showed that HA/TCP scaffolds seeded with skeletal stem cells improved ALP activities at higher porosities 28 days after implantation in mice (Aarvold et al. 2013). TCP (20%) incorporated poly(L-lactide-co-D,L-lactide) scaffolds with porosities ranging from 80 to 88% have shown higher neo-tissue formation following the utilization of scaffolds with large pores implanted in rabbit craniums (Roy et al. 2003). A literature survey revealed positive result of porosity on the new bone formation, in vivo. Nevertheless, there are few studies stating that porosity does not affect tissue formation (Amemiya et al. 2012).

The effect of mean pore size on osteogenesis has always been a subject of disparity. For instance, a research group reported the effect of mean pore size of collagen-glycosaminoglycan (CG) scaffolds ranging from 85 to 325 μm on osteoblast adhesion and proliferation 1 week after cell seeding. The results indicated that the maximum cell number was obtained in the scaffold with the largest pore size (325 μm). The authors asserted that the scaffolds with mean pore size of 325 μm are ideal for bone tissue engineering applications since pore sizes above 300 μm reduce cell aggregation around the scaffolds (Murphy et al. 2010). Akay et al. modified the surface of polyHIPE polymer (PHP) with HA and achieved noticeable levels of biomineralization by using primary rat osteoblasts after 28 and 35 days. However, evaluation of the effect of pore size showed that different pore sizes

(ranging from 40 to 100 μm) did not show such effects (Akay et al. 2004). Similarly, PCL scaffolds with pore sizes ranging from 0.5 to 1.5 mm, seeded with adipose-derived stem cells (ASCs), showed higher levels of mineralization as evidenced by von Kossa staining at larger pore sizes (Huri et al. 2014). Early in vivo experiments defined 100 μm as the minimum pore size of scaffold for successful bone tissue engineering applications. Subsequently, the optimum values for pore size were changed. In a series of experiments on calcium phosphate cement (CPC) porous scaffolds, the optimum pore size was shown to be in the range of 200 to 300 μm. This range favored bone formation as reflected by a suitable alkaline phosphatase (ALP) activity, better histological outcomes, and good mechanical properties (Zhao et al. 2017). However, for β-tricalcium phosphate (β-TCP) bioceramics, 400 μm was the best pore size. In vivo experiments confirmed fibrous tissue ingrowth and appropriate blood vessel formation at the pore size (of 400 μm) (Feng et al. 2011). Recently, Lu et al. explored the effect of pore and interconnection size on bone tissue-engineered scaffolds. They implanted hydroxyapatite (HA) bioceramic scaffolds into distal femoral condyle defects of 72 rabbits and observed considerable bone formation by increasing the interconnection size. The results showed increased bone formation at 4th week with pore sizes of ~400 μm, but no significant difference was observed with increasing cultivation time (24 weeks). Finally, the minimum interconnection size of 120 μm was proposed (Lu et al. 2016). Pore sizes in the range of 750–900 μm were also recommended for rapid angiogenesis, osteogenesis, and uniform distribution of new bone of hydroxyapatite (HA) scaffolds (Li et al. 2016). Similarly, the influence of biodegradation rate, pore size, and porosity on bone healing process was examined in magnesium phosphate (MgP) scaffolds. The degradation rate and bone regeneration match at pore sizes ranging from 25 to 53 μm. Implantation of these scaffolds exhibited improved calcification and lamellar structure in comparison to nonporous scaffolds due to their suitable porosity and biodegradation rate (Kim et al. 2016).

3.4 Surface Chemistry

Cell-scaffold interactions highly control the cellular functions. In particular, stem cell-scaffold interactions can affect the stem cell fate. Surface chemistry (i.e., hydrophilicity and surface charge) and topography alters cellular behaviors such as adhesion, migration, proliferation, and differentiation (Faghihi and Baghaban Eslaminejad 2014). Goat bone marrow stromal cells showed proliferation and differentiation in a 3D synthetic scaffold with tunable hydrophilicity properties (Escobar Ivirico et al. 2009). Role of hydrophilicity and hydrophobicity as a design criterion has been comprehensively discussed, and it was concluded that more hydrophobic scaffolds are more appropriate substrates for bone TE (Jansen et al. 2005).

3.5 Mechanical Properties

The use of graft or scaffold as a mechanical support is of crucial importance in bone healing approaches. The mechanical properties of scaffold and host tissue need to match since they determine the mechanotransduction (i.e., the cells respond to mechanical stimulation by converting mechanical signal to biochemical signals). Indeed, the growth and integration of the newly formed tissue are governed by mechanical properties of the scaffolds (Hing 2004). Mechanical properties lower than a threshold value cause failure of the scaffolds under in vivo conditions as shown by deformed tissue shapes. In addition, mechanical properties higher than a critical limit may decrease the elasticity of the scaffold under in vivo conditions. Moreover, application of the scaffolds with much higher mechanical properties of the neighboring bone also leads to application of low stress which therefore prevents the new bone formation and decreases density of adjacent bone. This phenomenon is called “stress shielding” and causes the bone resorption in the vicinity of the bone implants (Chanlalit et al. 2012).

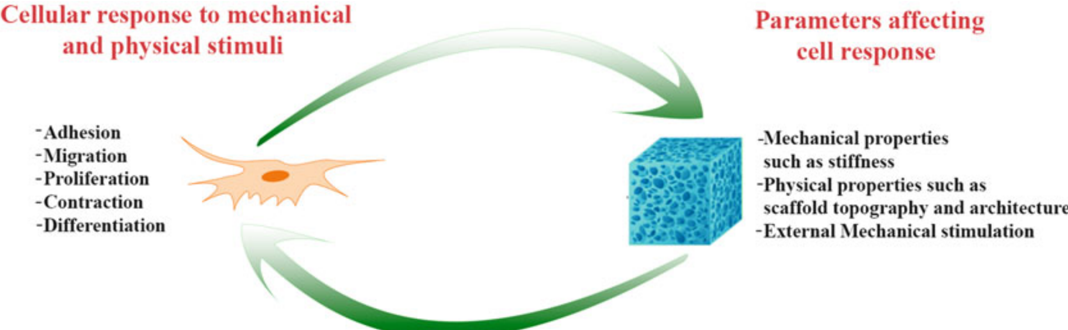

Fig. 2 Cell-scaffold reciprocal interactions

3.6 Osteoimmunomodulation

Inconsistency between in vitro and preclinical results was awkwardly ignored in traditional studies of bone tissue engineering strategies. Such a contradiction is believed to be caused by not considering the modulating properties of immune cells. Osteoimmunology demonstrates the importance of immune cells in controlling bone dynamic and cell-biomaterial interactions. The disagreements of in vivo and in vitro results may be avoided by considering the vital role of immunologic responses. Osteoimmunomodulation highlights the importance of the immune response in scaffold-based osteogenesis. Therefore, the next generation of bone tissue engineering strategies must consider osteoimmunomodulatory properties of biomaterials and scaffolds as a new determining factor (Rivera-Munoz 2015).

4 Effective Parameters in the Reciprocal Cell-Scaffold Interaction

Generally, selection of an appropriate source for both cell and scaffold strongly depends on the particular tissue that needs to be engineered. These challenges necessitate complete investigation of cell-scaffold interactions. The reciprocal biochemical and biomechanical interactions between cells and scaffolds determine cell functionality and final scaffold property within the designed tissue and eventually define the success rate of implantation in clinical settings (Fig. 2). (Ahearne 2014; Dado and Levenberg 2009; Murphy et al. 2013).

4.1 Cell-Mediated Remodeling of Scaffolds

Cells are able to generate their ECM through several mechanical and biochemical mechanisms. Numerous cellular processes such as adhesion, migration, contraction, degradation, and extracellular matrix deposition contribute to cell-mediated remodeling of scaffolds (Dado and Levenberg 2009). This remodeling depends on four factors, namely, (a) type of scaffold, (b) type of cells, (c) biochemical signaling molecules, and (d) mechanical signals. Effects of the cellular events on the scaffolds are demonstrated in various studies (Dado and Levenberg 2009).

Cell adhesion which is governed by cell surface receptors (e.g., integrin) and attachment to the scaffold ligands plays a vital role in cell-material mechano-interactions (Doyle and Yamada 2016). Functions of integrin can be categorized based on their role in cell attachment, cell migration, extracellular matrix remodeling, and cell signaling. It has been shown that increased cell adhesive moieties, such as collagen and glycosaminoglycan (GAGs) in a collagen-GAG scaffold, had a considerable influence on osteoblast activity (Murphy et al. 2010).

Cell migration and mobility depend on factors like contractile forces applied by the cell, availability of binding sites, degradation of matrix components, and scaffold matrix stiffness. Cell migration can be stimulated by three so-called mechanisms including chemotaxis, durotaxis, and mechanotaxis. Chemotaxis is defined as the movement of the cells in response to chemical stimulus such as growth factors. Durotaxis is a kind of cell migration which is directed via stiffness gradients. In this regard, most cells types commonly migrate toward higher stiffness. In mechanotaxis, movement of cell is governed by mechanical stimulators such as fluid shear stress. It is established that cells migrate in the direction of fluid flow (Dado and Levenberg 2009).

Cell contraction is the process of elongation and adhesion of cells and their spread on pore walls in the scaffolds. The cells then start to buckle the pores wall together that leads to scaffold contraction. It should be noted that cell contraction may cause 20–30% shrinkage in the final tissue-engineered construct which is not satisfactory, since this contraction may result in a construct that does not fits the defect site. Cell contraction may be controlled by degree and mechanism of cross-linking and the number of binding sites which is dependent on material concentration (Ahearne 2014).

Scaffold degradation by hydrolysis and enzymatic digestion is another mechanism of cell-mediated scaffold remodeling. The rate of scaffold degradation can be regulated by mechanical forces applied to the scaffold. Mineralization as the process of extracellular matrix deposition also affects the physical and mechanical properties of the scaffolds used for bone tissue engineering applications (Dado and Levenberg 2009).

4.2 Influence of Scaffold on Cellular Behaviors

Mechanical, structural, and chemical composition of the scaffold can also regulate the intracellular processes. Cells respond to different scaffold stiffness or matrix structure in different ways such as alteration in cell phenotype, cytoskeleton configuration, proliferation, and mobility. MSCs have shown enhanced proliferation rate on stiffer scaffolds, whereas proliferation of neural stem cells reduced by increasing scaffold stiffness (Leipzig and Shoichet 2009; Park et al. 2011). This kind of cell-dependent behavior may be related to the fact that cells would like to mimic their natural ECM. For instance, the mechanical properties of the native tissues vary from soft brain tissue (0.1 kPa) to pre-calcified bone (100 kPa) and rigid compact bone (20 GPa). Other factors such as spatial arrangement of scaffold, magnetic field alignment, viscoelastic behavior, and incorporation of nanoparticles can also affect the cellular behavior (Ahearne 2014).

Following application of force to cell-scaffold constructs, a series of events occur as follows: alteration of cytoskeleton configuration, disruption of cell nucleus, activation of ion channels, and phosphorylation. Activation of ion channels in turn leads to the release of signaling molecules that affect the behavior of adjacent cells. In order to mimic the natural environment of the desired tissue, bioreactors with distinct properties are designed. Generally, four kinds of bioreactors including spinner flasks bioreactors, perfusion bioreactors, rotating wall vessel bioreactors, rotating bed bioreactors, and biaxial rotating bioreactors have been used to study the effect of mechanical stimulation on MSC fate (Zhang et al. 2010; Zhao and Ma 2005). Several groups have investigated the effect of cyclic hydrostatic pressure on osteoprogenitor cells and demonstrated enhanced new bone formation (Liu et al. 2010). Also, induction of osteogenic differentiation and mineralization is reported following stimulation by fluid flow shear stress. This kind of stimulation is accomplished by pumping the fluid through the 3D scaffold by perfusion bioreactors. There are some systematic studies comparing several kinds of bioreactors used for bone tissue engineering (Zhang et al. 2010).

5 Cell-Loading Approaches

One of the crucial factors affecting the success rate of cell-scaffold based strategy is the efficient cell seeding approach. Considering the 3D culture system used in tissue engineering, seeding the cells onto the porous scaffolds is one of the common issues. Complicated structure of 3D scaffolds requires a robust method to support high number of attached cells, facilitate cell penetration into the scaffold homogenously, and provide reproducibility. Till now, several seeding techniques that can be generally categorized into static and dynamic seeding have been utilized to create effective constructs with their merits and demerits.

Static or passive seeding is a routine way of cell seeding which is done by directly placing cell suspension onto the scaffold or immersing the scaffold in cell containing medium and allow to infiltrate into the scaffold. This method has been found to be ineffective due to low rate of cell penetration and low seeding yield of about 10–25%. To overcome these drawbacks, static cell seeding can be carried out by biological glues such as fibrin and fibronectin which facilitate cell trapping within the scaffolds and increase cells attachment. We have also reported that the presence of collagen gel in the seeding medium can significantly enhance the efficiency of cell loading into the scaffold pores (Eslaminejad et al. 2009).

In contrast, dynamic seeding as an elaborate technique increases cell seeding efficiency, uniformity, and penetration into the scaffolds. Dynamic cell seeding is generally performed by application of external forces like low pressure, vacuum, and centrifugation. In this respect, several vessels have been used including spinner flask, vacuum chamber/perfused bioreactor, and orbital shaker (Griffon et al. 2005; Weinand et al. 2009). A number of studies have reported higher homogeneity achieved by vacuum seeding as a dynamic approach compared to static particularly for highly porous scaffolds (Yoshii et al. 2009). However, contradictory results were reported by Kuijer et al. as they observed higher homogeneous cell distribution in TCP scaffolds with lower porosity compared to high porous scaffold (Buizer et al. 2014). In another attempt, hMSCs were seeded onto HYAFF®-11 sponges quantitatively and homogeneously by vacuum loading technique with no negative effect on cell viability or chondrogenic potential (Solchaga et al. 2006). Such discrepancy may be related to the scaffold materials, structure, and methodology used for cell seeding.

Application of centrifugal forces has also been described as an efficient method in order to transfer cells into porous scaffolds (Beloti et al. 2009; Roh et al. 2007). Various cell types including hepatocyte, cardiomyocyte, and BMSCs have been efficiently seeded onto the porous scaffolds by these methods (Dar et al. 2002; Dvir-Ginzberg et al. 2003; Godbey et al. 2004; Yang et al. 2001). Application of short-time centrifugal force results in considerable amount of attached cells onto the scaffolds compared to prolonged spinner flask seeding or static seeding (Godbey et al. 2004).

The use of nanoparticles and magnetic forces is considered as alternative cell seeding methods. Cells labeled with magnetic nanoparticle were seeded onto a scaffold through application of external magnetic force. Magnetic force caused cells to infiltrate into the central parts of scaffolds with high efficiency and uniform distribution within the matrix (Shimizu et al. 2006).

Although all these methods resulted in encouraging results, more research is being done for discovery of a rapid, reproducible, and efficient approach (Wang et al. 2009). For example, Tan et al. developed a new method called “syringe-vacuum cell seeding” and achieved a homogenous distribution of cells through the scaffolds with a seeding efficiency of 60% (Tan et al. 2012). Moreover, (3D) bioprinting provides a sophisticated way of cell loading. This technique allows the distribution of various cells within supporting biomaterials at desired location. More recently, hiPSCs were printed using a nanofibrillated cellulose (NFC) composite bioink and co-printed with human chondrocytes resulting in formation of hyaline-like cartilaginous tissue (Nguyen et al. 2017).

Temporarily exposure of cell suspension to high temperature or high acceleration and deceleration may affect cell viability which should be improved in new generation of cell-printing approaches.

6 Cell-Loaded Constructs in Clinical and Preclinical Settings

Damage of musculoskeletal tissues including bone, cartilage, tendon, and ligament following tumor resection may result in large-sized defects that need to be reconstructed. This section reviews the clinical and preclinical studies done to heal large boney non-unions or large tissue voids using cell-seeded constructs. Moreover, potential of preloaded scaffolds and existing shortcoming for prospective therapeutic applications are discussed.

In terms of bone tissue regeneration, the first attempt was made by Goshima et al. who demonstrated deposition of mineralized matrix and bone formation in BMSC-seeded porous bioceramic scaffolds after subcutaneous implantation into immunocompromised mice (Goshima et al. 1991b). Afterward, a number of research groups subcutaneously implanted similar scaffolds seeded with BMSCs from different species, in syngeneic rats (Goshima et al. 1991a), immunodeficient mice (Kadiyala et al. 1997), or within small experimentally induced osseous defects (Ohgushi et al. 1989), and observed the same outcome. However, these findings did not provide any information about the integrity of old bone and newly formed one.

The regenerative ability of cell-loaded constructs was assessed in critical-sized bone defects. BMSC-embedded calcium alginate constructs were implanted to regenerate sheep calvarial defects (diameter 20 mm). Bone repair occurred in the defect with fabricated construct 18 weeks postimplantation, while fibrous tissues were only formed in control group and no sign of bone healing was observed (Shang et al. 2001). It has been reported that BMSC-seeded poly(lactic-co-glycolic acid) (PLGA) scaffolds implanted into mandible defects of a rabbit model showed significant amount of newly formed bone 4 and 8 weeks postimplantation (Liu et al. 2014). Similarly, engineered bone constructs that included calcium phosphate scaffold seeded with BMSCs were used in mandibular bone defects in a canine model. Newly formed bone was detected 4 weeks postsurgery, and the union bone was formed after 32 weeks (Yuan et al. 2007). In fact, the efficiency of cell-loaded in comparison to cell-free constructs was examined in numerous studies. Bruder et al. implanted porous bioceramic constructs in both forms of non-seeded and seeded with BMSCs into critical-sized (21 mm) segmental defects in dog femora. Radiographic union was established rapidly at the host bone-implants interfacial zone in the cell-seeded group. In contrast, several fractures occurred in the untreated defects, which progressively increased. After 4 months, pore areas were filled with 39.9% and 24% of bone in the cell-loaded constructs and the untreated ones, respectively (Bruder et al. 1998). Another study has assessed the ability of BMSC-loaded hydroxyapatite ceramic to regenerate critical-sized bone defect in a sheep model (Kon et al. 2000).

Following the encouraging advancements in using tissue-engineered constructs in animal models, several groups have reported the successful repair of bone defects in clinical settings (Marcacci et al. 2007; Quarto et al. 2001; Vacanti et al. 2001). Primary attempts used custom-made porous HA ceramic scaffolds to match the bone defects in terms of size and shape. The treatment was performed on four patients aging between 16 and 41 years old who experienced the failure of alternative more “conventional” surgical therapies. The treatment process in all patients was followed up at various time points postsurgery and manifested no major complications or complaints. Radiographs and computed tomography (CT) scans revealed the callus formation and host-implant fusion at interface zone 2 months postimplantation. Long-term follow-ups first disclosed complete implant-host bone interaction (5–7 months after surgery) and eventually showed functional limb regeneration (between 6 and 12 months). Also, a 6–7-year

follow-up proved a substantial integrity between host tissue and newly formed bone in two of the patients (Marcacci et al. 2007). In another clinical trial which mainly focused on the treatment of patients with craniomaxillofacial bone defects, autologous BMSCs seeded onto partially demineralized bone matrix (pDBM) were implanted into cranial defects of 11 patients (Chai et al. 2003). CT scan and histology analysis proved the necessity of osteogenically induced BMSCs for stable bone formation in humans, as complete resorption was observed in non-cell-seeded pDBM group. Formation of cancellous bone and expression of osteocalcin and osteopontin by the engineered human bone were evidenced by taking biopsy after 18 months.

Among enormous studies done on bone tissue regeneration of large defects, a few number of preclinical and clinical trials have been exclusively reported the reconstruction of tumor resection defects. Morishita et al. in 2006 isolated MSCs from three patients' bone marrow and subsequently loaded the BMSCs on porous HA scaffolds. BMSCs were committed to osteogenic lineage and implanted into the bone defect after tumor removal. Radiographs and CT images revealed the incorporation of MSC-HA construct within host bone 3 months postoperation and a higher mechanical strength of treated defect after implantation (Morishita et al. 2006). A clinical trial was also carried out to regenerate large-sized defect following nonmalignant bone tumor resection. A combination of pre-differentiated autologous BMSCs and porous hydroxyapatite ceramic scaffolds was implanted into the defect, and safety and efficiency of treatment approach were assessed (Myoui and Yoshikawa 2008). These studies support the constructive role of MSC-loaded constructs in clinical settings; however, there are several other issues concerning this novel strategy that should be addressed in future investigations (Table 1).

In case of cartilage, a great number of attempts have been made to repair the lesions (Table 2). Combination of cells (particularly chondrocytes) and scaffolds has emerged as an efficient therapeutic strategy called "matrix-assisted chondrocyte implantation (MACI)." Chondrocytes embedded in collagen gel were transplanted into full-thickness articular cartilage defects in rabbits. The defects filled with hyaline cartilage, specifically generated collagen type II, 24 weeks postsurgery (Wakitani et al. 1989). Since major complications have been reported for chondrocytes' harvesting, expansion, and culture, other cell sources particularly MSCs have attracted considerable attention. Transplantation of autologous MSCs combined with hyaluronic acid gel sponges into full-thickness osteochondral defects of the knee in a rabbit model led to cartilage-like tissue formation. Well-repaired cartilage tissue that resembles the healthy articular cartilage structure was obtained in cell-loaded groups, 12 weeks postsurgery (Kayakabe et al. 2006). Encapsulation of BMSCs in oligo(poly(ethylene glycol) fumarate) (OPF)/gelatin microparticle (GMP) hydrogel composites stimulates the subchondral bone formation without provoking inflammation. The type of TGF-b1-loaded construct did not affect the cartilage morphology or MSCs differentiations (Guo et al. 2010). Simplified scaffold-MSC constructs without any growth factors or gene transfer may literally be useful in clinical applications. In an attempt made by Xue et al., MSCs loaded onto porous nano-hydroxyapatite/PLGA scaffolds directed the regeneration of articular lesion in rats. Monitoring fluorescent-labeled MSCs revealed that transplanted MSCs involved in defect regeneration were alive 12 weeks postoperation without any growth factors or gene transfer (Xue et al. 2010).

Another point that should be mentioned is whether various cell types have different chondrogenic capabilities, in vivo. Koga et al. conducted a study to address chondrogenic potential of MSCs derived from bone marrow, synovium, adipose tissue, and muscle of adult rabbits embedded in collagen gel. They found that synovium- and bone marrow-MSCs had greater ability to commit chondrogenic lineage compared to adipose- and muscle-MSCs in full-thickness cartilage defects. However, synovium-derived MSCs (SMSCs) possess higher proliferative potential resulting in production of higher amounts of cartilaginous matrix (Koga et al. 2008). Likewise, a comparison made among chondrocytes, MSCs, fibroblasts, and

Table 1 Clinical and preclinical studies using MSC-loaded scaffolds for bone regeneration

No.	Cell sources	Scaffold	Species	Repair site	Results	References
1	BMSCs	Calcium alginate	Sheep	Cranium	Neo-bone formation occurred in the defects treated with BMSC scaffold 8 weeks postsurgery. After 18 weeks, the defect of experimental group was almost completely repaired as evidenced by μ-CT	Shang et al. (2001)
2	Epithelial cells-BMSCs	PLGA	Rabbit	Mandible	The results showed that MSCs survived on the scaffold and could promote bone formation. Larger area of defects was repaired in MSC-EC-PLGA group	Liu et al. (2014)
3	BMSCs	Beta-TCP	Canine	Mandible	Treatment of mandibular defect with BMSCs/beta-TCP resulted in new bone formation 4 weeks postimplantation and bony union after 32 weeks with high degree of biomechanical strength	Yuan et al. (2007)
4	BMSCs	Hydroxyapatite	Sheep	Tibia	Newly formed bone was detected within the internal macropore space and outer surface of HA substrates, while in cell-free groups, bone formation was limited mostly to the outer surface. Combination of BMSC with HAC-based carriers resulted in faster bone repair compared to HA alone	Kon et al. (2000)
5	BMSCs	Calcium alginate	Canine	Alveolar	Histological analysis revealed the formation of bone nodules 4 weeks after implantation, and this structure is replaced by mature bone after 12 weeks	Weng et al. (2006)
6	BMSCs	Hydroxyapatite and beta-TCP	Canine	Femora	Both woven and lamellar bone were detected in the MSC-scaffold group in larger amount compared to non-seeded group. Callus did not develop around the cortex of the host bone or around the defect	Bruder et al. (1998)
7	MSCs	Calcium phosphate ceramics	Rat	Femora	Combination of BMSCs increased the osteochondral union from 30% in group that calcium phosphate ceramics was used alone to 70%	Ohgushi et al. (1989)

(continued)

Table 1 (continued)

No.	Cell sources	Scaffold	Species	Repair site	Results	References
8	BMSCs	Platelet-rich plasma (PRP) and fibrin	Canine	Mandible	Histological and histomorphometric analysis showed that BMSCs/fibrin/PRP enhanced bone regeneration around dental implants	Ito et al. (2006)
9	BMSCs	Hydroxyapatite ceramic	Human	Diaphysis	New bone formation inside the bioceramic pores and vascular ingrowth was observed. Long-term follow-up showed progressive integration of the implant-host bone, with no late fracture at implantation site	Marcacci et al. (2007)
10	BMSCs	Demineralized allogenic bone matrix	Human	Craniomaxillofacial	Implanted BMSCs caused new bone formation in humans through endochondral ossification in 3–6 months coincidence with scaffold degradation. Engineered bone was stable for 18 months	Chai et al. (2003)
11	BMSCs	Hydroxyapatite/tricalcium phosphate	Canine	Femora	Implantation of autologous BMSC combined with ceramic scaffold mediated osteogenesis in a canine model	Kadiyala et al. (1997)
12	BMSCs	Calcium phosphate ceramics	Rat	Subcutaneous	This study provides primary data in cell-scaffold approach. BMSCs in conjunction with ceramics resulted in considerable osteogenic potential and bone formation within the pore regions of ceramics 2 weeks after implantation	Goshima et al. (1991a)
13	Human BMSCs, ovine BMSCs	Mineralized collagen	Sheep	Tibia	Radiology and histological analysis indicated that the autologous transplantation of ovine BMSCs enhanced bone regeneration compared to xenogeneic hBMSCs	Niemeyer et al. (2010)
14	Endothelial cells-BMSCs	PLGA	Nude rat	Calvaria	Early mineralization occurred in experimental group as evidenced by μ-CT	Kaigler et al. (2006)
15	BMSCs	Hydroxyapatite ceramic	Human	Diaphysis	Abundant callus formation and adequate integration at the implant-host tissue surface occurred 5–7 months postsurgery	Marcacci et al. (2007)

(continued)

Table 1 (continued)

No.	Cell sources	Scaffold	Species	Repair site	Results	References
16	BMSCs	Hydroxyapatite/ beta-TCP	Human	Posterior maxilla	Histological analysis showed that MSC- seeded biomaterials resulted in enhanced bone formation and osseointegration in the maxillary sinus area	Shayesteh et al. (2008)
17	ADMSCs	Beta-TCP	Human	Bone flap	The flap bone regenerated and formed a structure that resembled mature maxillary bone after 8 months	Mesimaki et al. (2009)
18	Dental pulp stem/ progenitor cells	Collagen sponge	Human	Alveolar	The results demonstrated regeneration of periodontal tissue 3 months following ADMSC/collagen substitute implantation	d'Aquino et al. (2009)

Table 2 Clinical and preclinical studies describing MSC-loaded scaffolds for cartilage regeneration

No.	Cell sources	Scaffold	Species	Repair site	Results	References
1	Chondrocyte	Fibrin	Horse	Full-thickness cartilage defect	The expression level of Col type II was upregulated, healing of cartilage lesions promoted, and integration with surrounding cartilage improved. The expression of Col type I and low amount of GAGs were related drawback	Fortier et al. (2002)
2	BMSCs	Fibrin clot	Rabbit	Osteochondral defects in knee joint	Regenerated tissue highly resembled the biomechanical and structure of native cartilage tissue	Berninger et al. (2013)
3	BMSCs	Hyaluronic acid	Rabbit	Osteochondral defects	The results indicated that the defect treated with BMSC-seeded scaffold regenerated as it is highly similar to articular cartilage in the surrounding structure after 12 weeks	Kayakabe et al. (2006)
4	BMSCs	PLA-polyglycolic acid (PGA)	Pigs	Osteochondral defects	Histological analysis demonstrated formation of hyaline cartilage and cancellous bone as well as better restoration level in experimental group	Zhou et al. (2006)
5	BMSCs	Oligo(poly (ethylene glycol) fumarate)/ gelatin microparticles	Rabbit	Osteochondral defects	Neo-tissue formed and filled the defect along with degradation of OPF scaffold. Addition of BMSCs provoked the subchondral bone formation	Guo et al. (2010)

(continued)

Table 2 (continued)

No.	Cell sources	Scaffold	Species	Repair site	Results	References
6	BMSCs, fibroblasts, chondrocytes, and hUCMSCs	PLA	Rabbit	Full-thickness cartilage defect	Hyaline-like cartilage were found in defects treated with MSC and chondrocyte, while fibroblast and UCMSC groups formed fibrocartilage and thin layer of hyaline cartilage, respectively	Yan and Yu (2007)
7	BMSCs	Collagen type I	Rabbit	Full-thickness articular cartilage defects	Defects did not fully repaired, but the implantation of autologous CDMP1-transfected BMMCs improved restoration of hyaline cartilage at surface zone	Katayama et al. (2004)
8	BMSCs	Fibrin hydrogel	Mice	Back subcutis	Higher level of type II and proteoglycan were detected in BMSC/fibrin construct. On the other hand, the expression level of collagen type I was downregulated over 4 weeks in the presence of TGF-b3 both in vitro and in vivo	Park et al. (2009)
9	BMSCs	Poly(ethylene) oxide diacrylate	Mice	Subcutaneous injection	The expression level of chondrogenic-related genes as well as proteoglycan and collagen II production increased, but the col type I expression level was downregulated	Sharma et al. (2007)
10	BMSCs	PCL)/TCP	Rabbit	Osteochondral defects	BMSC-seeded group promoted new osteochondral tissue formation which in turn led to scaffold-host integration from 3 to 6 months. Biomechanical properties of neo-tissue was similar to that of normal cartilage	(Shao et al. 2006)
11	BMSCs	PLGA/Nano-hydroxyapatite	Rat	Osteochondral defects	The findings revealed that the defect treated with PLGA/NHA-MSCs filled with smooth and hyaline-like cartilage. Abundant glycosaminoglycan and col type II yet low amount of col type I were detected 12 weeks postimplantation	Xue et al. (2010)
12	UCMSCs	Collagen	Human	Cartilage defect	MSCs provided a sufficient support to facilitate cartilage formation. They also promoted angiogenesis through paracrine effects	Sadlik et al. (2017)

human umbilical cord blood (hUCB) seeded onto a polylactic acid (PLA) scaffold revealed higher regenerative abilities of chondrocytes and MSCs in full-thickness cartilage defects in a rabbit model. Histological analysis confirmed formation of hyaline-like cartilage tissue following chondrocytes or MSCs transplantation. Nevertheless, the tissue repaired using MSCs showed higher levels of cell arrangement, subchondral bone remodeling, and integration with surrounding cartilages compared to those repaired by chondrocytes (Yan and Yu 2007). MSCs derived from umbilical cord Wharton's jelly (UCMSCs) which embedded in collagen scaffold also resulted in regeneration of cartilage lesions (Sadlik et al. 2017). All these findings reflect the excellence of administration of MSCs combined with biomaterials for cartilage tissue regeneration.

Functional repair of either cartilage lesions or osteochondral defects in large animal models are of great importance due to their similarity to humans. Indeed, satisfactory outcomes observed in large animal models pave the way toward clinical trials. Several important issues related to articular osteochondral defects including role of in vitro chondrogenic induction, differentiation and distribution of exogenous MSCs in articular environment, and their contribution to healing process were also addressed. A combination of autologous BMSCs and biodegradable polymers was employed to repair osteochondral defects in a porcine model. Retrovirally GFP-labeled BMSCs successfully underwent chondrogenic and osteogenic differentiation and led to hyaline cartilage and subchondral bone tissue formation in articular osteochondral defects (Zhou et al. 2006). More preclinical efforts are still required to address the complications (e.g., tendency of MSCs to undergo hypertrophy) that hamper the clinical use of these constructs.

Tendon as one of the musculoskeletal parts may be damaged following tumor resection. Moreover, tendon injuries (called "tendinopathy") represent a common clinical problem that annually affect 30 million people, worldwide (Maffulli et al. 2003). Tendinopathy occurs as a result of trauma, abuse, and aging and is typically characterized by the presence of pain and inflammation along with dysfunction (Raikin et al. 2013; Sharma and Maffulli 2005). Tissue engineering techniques have been successful in the treatment of tendon and ligament defects. Despite various strategies such as cell therapy with BMSCs (Chong et al. 2007) that have been used for tendon regeneration, cell-scaffold constructs have given promising results (Table 3). Implantation of MSC-seeded organized collagen constructs into a large tendon defect accelerated tendon repair and improved tissue biomechanics and matrix organization compared to a contralateral, natural repair using suture alone (Young et al. 1998). Similarly, the structure and biomechanical function of a defect of Achilles tendon were restored using knitted PLGA loaded with BMSCs in a rabbit model (Vaquette et al. 2010). As an effective way, bioactive agents combined with scaffolds could be employed to accelerate the regeneration of tendon injuries. Supplementation of tendon hydrogel with both PRP and adipose MSCs enhances tendon healing process (Chiou et al. 2015).

Tendon attachment to porous calcium hydroxyapatite ceramics (CHC) was also enhanced when BMSCs were loaded onto the scaffold. This study showed marked bone formation around the implanted tendon/CHC/BMSC constructs in knee defect of a rabbit model (Omae et al. 2007). Chong et al. treated the tendon injury using transplantation of allogenic BMSCs in fibrin carrier (Chong et al. 2007). Histological and biomechanical analysis showed organized structures and improved modulus in the treatment group. These studies confirmed the crucial role of MSCs in tissue regeneration. Nevertheless, Pietschmann et al. compared the regenerative potential of MSCs and tenocytes on PGA and collagen type I scaffolds implanted in a critical-sized tendon defect. They observed that biomechanical strength and histologic properties of newly regenerated tissue were increased by tenocytes when compared to MSCs or empty scaffolds (Pietschmann et al. 2013).

Apart from all applied strategy and promising results, reconstruction of tendon with proper mechanical strength and functions still demands further investigations.

Table 3 Preclinical studies describing MSC-loaded scaffolds for tendon regeneration

No.	Cell sources	Scaffold	Species	Results	References
1	BMSCs	Suture coated with ICAM-1 and poly-L-lysine	Rat	The BMSC-coated suture group improved tendon healing with greater mechanical strength compared to the suture-only group	Yao et al. (2012)
2	BMSCs	Fibrin glue	Rabbit	Collagen fiber was well-organized in experimental group at 3 weeks. No differences with regard to the gross morphology were detected between the two groups of the tendons. Labeled MSCs were present in the intratendinous region for at least 6 weeks	Chong et al. (2007)
3	BMSCs Tenocytes	PGA and collagen type I	Rat	This study revealed ectopic ossification in all groups. However, biomechanical properties and degree of ectopic ossification were significantly higher in tenocytes seeded compared to the BMSC-transplanted and the scaffold-only groups	Pietschmann et al. (2013)
4	BMSCs	Silk-fibroin-PLGA mesh filled with collagen type I	Rabbit	Expression level of col type I gene and a uniformly dense distribution of collagen fibers confirmed formation of tendon-like tissues. The maximum load of the regenerated Achilles tendon achieved in MSC-seeded group	Zhang et al. (2015)
5	BMSCs	Collagen gel	Rabbit	MSC-collagen constructs accelerated tendon repair and improved tissue biomechanics and matrix organization compared to the control tissues	Young et al. (1998)
6	BMSCs	PLGA alginate	Rabbit	Elasticity of the regenerated tendons was significantly higher in MSC-seeded scaffold (60%) in comparison with control group (40%)	Vaquette et al. (2010)
7	BMSCs	Knitted PLGA and fibrin glue	Rabbit	Implantation of knitted PLGA scaffold loaded with BMSCs resulted in higher tensile stiffness and modulus of regenerated tissue with high amount of col type I and type III fibers	Ouyang et al. (2003)
8	ADMSCs	Tendon hydrogel +/− PRP	Rat	Ultimate load was increased as a result of transplantation of ADMSCs, PRP, or both in hydrogel compared to hydrogel alone. ECM formation and cellularity were increased in PRP and ADMSCs, respectively, as evidenced by histological analysis	(Chiou et al. 2015)
9	BMSCs	Polypropylene surgical mesh-PGA-alginate hydrogel	Rat	Dense and parallel collagen bundles, abundant col type I along with reduced vascularity, were observed in BMSC-loaded mesh group	Schon et al. (2014)
10	BMSCs	Fibrin glue	Horse	Both BMSC and bone marrow mononuclear cells (BMMNCS) improve tendon healing as reflected by high expression level of cartilage oligomeric matrix protein (COMP) and col type I, but low levels of col type III	Crovace et al. (2010)

7 Concluding Remarks

To avoid amputation of a cancerous bone, surgery is a logical way of removal of malignant tumor, but in turn it causes a large-sized defect that should be regenerated. Developing a clinically effective therapeutic approach to fill and regenerate the lost bone mass following tumor resection is still challenging. Efforts made to overcome the shortcomings of traditional approaches led to discovery of novel technology of TE that can potentially engineer all types of tissue. Among various

strategies employed for TE, cell-loaded scaffolds seem to be an appealing approach to repair critical-sized defect. This strategy has been extensively studied, in vitro and in vivo. Nevertheless, the efficacy and safety of this approach for repairing defects after tumor resection are still unclear. A few studies have been done in clinical settings, and favorable short-term outcomes have been achieved, but long-term effects are still unknown.

There are several issues concerning regeneration of these defects that should be taken into account. First of all, tumor resection usually involves removal of bone and surrounded tissues like cartilage and muscle which complicates the regeneration process and highlights the importance of interfacial tissue engineering. To date, several studies have been conducted to address the feasibility of two or more tissues, synchronously. For example, hybrid scaffolds containing two or more layers or continuous gradients hold great promise for regeneration of these tissues. Shao et al. fabricated fully interconnected porous PCL-TCP scaffolds to repair osteochondral defects in a rabbit model (Shao et al. 2006). Their findings revealed that the combination of BMSC and hybrid scaffold substantially improved bone and cartilage regeneration (Bailey et al. 2013; Pobloth et al. 2017). However, due to complexity of tissue-tissue interface and the parameters involved in this process, more investigations are required.

The most important issue is whether the risk of tumor recurrence at the site of tumor resection still exits or not. Therefore, any strategy, in particular, any cells, bioactive molecules, and materials must be used with caution, since they might potentially provoke the regrowth and development of malignant tumor. In this regard, administration of MSCs is the subject of controversy, as they behave unpredictably in different microenvironments. Tremendous studies have stated that MSCs are not tumorigenic; however, there are a few studies that verified this feature in cancerous tissues (Hernigou et al. 2014). The commitment of MSCs into distinct cell fate (e.g., osteoblastic lineage) before implantation reduces the risk of tumorigenicity and promotes the osteogenic activity. In addition to all drawbacks mentioned for MSCs like lack of a specific biomarker, age-related growth, and genetic instability, biosafety of autologous MSCs isolated cell from patients suffering from bone cancer should also be addressed. The other point that should be contemplated in TE of defects after tumor resection is vasculogenesis. Vasculogenesis has a stimulatory effect on tumor cell growth, thus scaffolds containing angiogenic factors must be applied with special care. Combination of scaffolds with anticancer drugs may also be considered as a rational approach.

Finally, successful application of tissue engineering concepts particularly cell-loaded scaffolds for patients suffering from malignant bone tumors requires more rigorous preclinical investigations which particularly consider tumor-promoting factors and long-term follow-up in large animal models.

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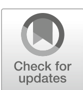

In Vitro Production of Cartilage Tissue from Rabbit Bone Marrow-Derived Mesenchymal Stem Cells and Polycaprolactone Scaffold

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Abstract

In vitro production of tissues or tissue engineering is a promising approach to produce artificial tissues for regenerative medicine. There are at least three important components of tissue engineering, including stem cells, scaffolds and growth factors. This study aimed to produce cartilage tissues in vitro from culture and chondrogenic differentiation of rabbit bone marrow-derived mesenchymal stem cells (BMMSCs), induced by chondrogenesis medium, on biodegradable polycaprolactone (PCL) scaffolds. BMMSCs were isolated from rabbit bone marrow according to the standard protocol. The adherence, proliferation and differentiation of BMMSCs on scaffolds were investigated using two scaffold systems: PCL scaffolds and collagen-coated PCL (PCL/col) scaffolds. The results showed that BMMSCs could attach and grow on both PCL and PCL/col scaffolds. However, the adhesion efficacy of BMMSCs on the PCL/col scaffolds was significantly better than on PCL scaffolds. Under induced conditions, BMMSCs on PLC/col scaffolds showed increased aggrecan accumulation and upregulated expression of chondrogenesis-associated genes (e.g. *collagen type II*, *collagen type I*, *aggrecan* and *collagen type X*) after 3, 7, 21 and 28 days of induction. These in vitro cartilage tissues could form mature chondrocyte-like cells after they were grafted into rabbits. The results suggest that use of BMMSCs in combination with polycaprolactone scaffolds and

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chondrogenesis medium can be a way to form in vitro cartilage tissue.

Keywords

Cartilage tissue engineering · Engineered cartilage · Polycaprolactone scaffolds · Regenerative medicine · Tissue engineering

Abbreviations

BMMSCs	Bone marrow-derived mesenchymal stem cells
CD	Cluster of differentiation
ECM	Extracellular matrix
GFP	Green fluorescent protein
MSC	Mesenchymal stem cell
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PCL/col	Collagen-coated PCL
PCR	Polymer chain reaction

1 Introduction

Cartilage tissue is present in various regions in the human body such as the ear, nose, ribs and joints. Cartilage tissue plays an important role for the body in structural support, maintenance of shape and reduction of friction on the joint head (Hall 2012). The lack of vascularization is a differentiating characteristic of this tissue from other tissues (Oseni et al. 2011). Also, for this reason in vivo regeneration of cartilage is limited after damage caused by trauma or degenerative diseases. Therefore, a number of studies have tried to produce cartilage tissue in vitro for the purposes of regenerative medicine.

In general, in vitro tissues can be formed by three components which include stem cells (which provide the cellular component of tissues), scaffolds (which provide the extracellular matrix for cells) and growth factors or inducible factors (which stimulate the adherence, proliferation and differentiation of stem cells inside the scaffold) (Portocarrero et al. 2013). There are different kinds of stem cells used in tissue engineering, but mesenchymal stem cells are the most popular stem cells, especially for generating cartilage tissue.

Mesenchymal stem cells can be collected from a variety of tissue resources such as bone marrow, adipose tissue, peripheral blood and umbilical cord blood (Kern et al. 2006; Shi and Gronthos 2003; Zhu et al. 2012). They are classified as multipotent cells with mesenchymal tri-lineage differentiation capacity, plastic-adherent ability, fibroblast-like morphology and specific markers (CD90+, CD44+, CD105+, CD73+, CD14−, CD34−, CD45−, etc.) (Dominici et al. 2006). In addition, MSCs have properties of immunoregulatory cells (Aggarwal and Pittenger 2005; Puissant et al. 2005; Yanez et al. 2006). In previous studies, adipose-derived mesenchymal stem cells (ADSCs) were used to promote angiogenesis in ischemic treatment (Vu et al. 2016, 2017), while bone marrow-derived mesenchymal stem cells (BMMSCs) were known to enhance the chondrogenic transcriptional programme (Reinisch et al. 2015; Zhu et al. 2012). Therefore, in this study, BMMSCs were used to produce cartilage tissue.

Scaffolds are an essential component of tissue engineering. Polycaprolactone (PCL) was first created from polymerization of ε-caprolactone monomer by Crother and colleagues in 1930 (Van Natta et al. 1934). This polymer is currently being used as a popular implant material. Because of its biodegradability, biocompatibility and non-cytotoxicity, PCL is being considered as a suitable material for scaffolds in cartilage tissue engineering applications (Woodruff and Hutmacher 2010). Woodward et al. showed that scaffolds are degraded via polymer hydrolysis via stages; first, ester links are randomly cleaved by non-enzyme hydrolysis and PCL fragments digested by intracellular phagosomes, and, second, products of PCL scaffold degradation are

emitted from the body via acid citric process (Woodward et al. 1985). Previous studies have demonstrated that the pore size of scaffolds can affect cell proliferation, adherence and survival (O'Brien et al. 2005). Suitable pore size of scaffolds allows for a suitable microenvironment necessary for nutrient uptake and removal of wastes (Karande et al. 2004). Besides, cell differentiation is also affected by pore size; for example, pore size ranging from 350 nm to 500 nm is suitable for bone and cartilage regeneration (Hutmacher 2000; Roosa et al. 2010).

The final component of tissue engineering is the use of growth factors and inducible factors. These factors help stem cells adhere, proliferate and differentiate into functional cells. Indeed, chondrogenesis of mesenchymal stem cells involves three main stages, including the formation of prechondrogenic condensations, the differentiation into chondrocytes and the maturation (Pizette and Niswander 2000). At the initiation stage of chondrogenesis, mesenchymal stem cells are recruited, migrated, proliferated and condensed. This process is stimulated by signals from cell-cell and cell-matrix interactions, such as transformed growth factor beta (TGF-β), fibroblast growth factor (FGF) and bone morphogenetic proteins (BMP) (Dexheimer et al. 2016; Minina et al. 2002; Pogue and Lyons 2006). These signals also enhance expression of adherent molecules (N-CAM, N-cadherin), formation of gap junctions and change of cytoskeletal architecture and cell proliferation. In this stage, extracellular matrix proteins, such as collagen type I, collagen type II, COMP and thrombospondins, are displayed (Tuan 2003). The differentiation of chondroprogenitors is a subsequent stage of skeletogenesis development and is identified by presentation of *collagen type II*, *collagen type XI*, *collagen type IX* and *aggrecan* (Yamashita et al. 2010). Among the earliest markers of cells undergoing condensation is the nuclear transcription factor *Sox9*. *Sox9* plays an important role in the expression of cartilage-matrix protein *Col2A1*. In the late stage, *collagen type X* and a positive regular factor *Runx2* are expressed as makers for chondrocyte maturation (Goldring et al. 2006).

In this study, we aimed to evaluate the in vitro formation of cartilage tissue of BMMSCs on PCL scaffolds with or without collagen coating under the same inducible conditions.

2 Materials-Methods

2.1 Fabrication of Biodegradable Scaffolds

The PCL scaffold was fabricated using PCL filament and a three-dimensional (3D) desktop printer IAMI2 that had been developed based on fused filament fabrication (FFF) technology. The PCL filament was melted with a heater then the scaffold formed by an extruder. The 3D scaffold model was designed using specific design programmes, such as CAD and CT/MRI. In others to build the resource data for 3D fabrication making, all files were transformed into stereolithography (STL) file format. After that, the open source slicer programme was used in the next steps to build G-code data. The data resource was then transferred to the 3D printer to build the scaffold.

The PCL filament was extruded line by line to create a 2D layer and then layer by layer to create a 3D structure. Once a layer was formed, the dispenser moved up vertically in Z-direction by a layer height of 200 μm. In addition, after each layer was completed, the dispensed lines would turn 90° to form scaffold architectural patterns which resulted in a 90° angle between the lines of the current layer and the last layer. The nozzle diameter was designed to be 200 μm. The extruder temperature and deposition speed were set at 85 °C and 210 mm/min, respectively. Also, the bed temperature was set at 55 °C for the first five layers. After that, the temperature was reduced to 52 °C by the Peltier cooler system.

The PCL scaffold was then sterilized by steeping in 70° ethanol overnight. The scaffolds were washed with PBS twice and exposed under UV light for 30 min. To enhance cell adhesion, they were treated with 0.025 mg/ml collagen (Sigma-Aldrich, St. Louis, MO, USA) and then dried naturally.

2.2 Isolation and Characterization of Rabbit Bone Marrow-Derived Mesenchymal Stem Cells (BMMSCs)

Bone marrow of New Zealand rabbits were collected after anaesthesia. All manipulations on rabbits were approved by Institutional Animal Ethics Committee (Stem Cell Institute, University of Science, VNUHCM, Ho Chi Minh City, Vietnam). Then the bone marrow was mixed with coagulated substance. The mixture was diluted with DMEM medium (Sigma-Aldrich, Louis St, MO) and centrifuged at 2500 rpm for 5 min to remove coagulated substance. The cell pellet was suspended with MSCCult (Regenmedlab Ltd., Ho Chi Minh City, Vietnam) and then divided to T25 flasks. The flasks were then incubated at 37 °C in a 5% CO_2 incubator. After 7 days, nonadhesive cells were removed by replenishing the medium. When the adherent cell density reached 70–80% confluency in the flasks, candidate BMMSCs were passaged with 0.25% trypsin/EDTA (Sigma-Aldrich, Louis St, MO).

The capacity of BMMSCs to differentiate into adipocytes, chondrocytes and osteocytes was evaluated using the StemPro® Adipogenesis, Chondrogenesis and Osteogenesis Differentiation Kits (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The lipid deposit was assessed by Oil Red O staining (Sigma-Aldrich, Louis St, MO). The expression of cartilage-specific proteoglycan was detected by Alcian Blue (Sigma-Aldrich, Louis St, MO) staining. Extracellular calcium deposits to investigate osteocyte formation were evaluated using Alizarin Red S (Sigma-Aldrich, Louis St, MO) staining.

2.3 Production of BMMSCs Expressing Green Fluorescent Protein (GFP-BMMSCs)

Rabbit BMMSCs were resuspended in MSCCult medium supplemented with 8 μg/ml hexadimethrine bromide (Sigma-Aldrich, Louis St, MO) and then seeded into 6-well plates at a density of 10^4 cells/well. 2 μl of copGFP control lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA) containing 10^4 infectious units of virus (IFU) was added to each well. After 3 days, selection medium containing MSCCult (RegenmedLab) supplied with 2 μg/ml puromycin (Sigma-Aldrich) was replenished to select for GFP expressing BMMSCs.

2.4 Seeding BMMSCs onto PCL Scaffolds

Scaffolds (3 mm × 3 mm) were placed individually in 0.2 ml tubes. GFP-BMMSCs were seeded onto the scaffolds after suspending to a concentration of 2×10^5 cells/100 μl of MSCCult (RegenmedLab). Cell-seeded scaffolds were cultured at 37 °C, 5% CO_2.

2.5 Cell Proliferation Assay

The proliferation ability of GFP-BMMSCs on PCL and collagen-coated PCL (PCL/col) scaffolds were assessed. Based on the analytical principle of colorimetric quantity, thiazolyl blue tetrazolium blue (MTT) was used to evaluate cell proliferation. The GFP-BMMSC suspension was seeded on the scaffold (in the tubes). After adherence, culture medium was refreshed. 10 μl of 5 mg/ml MTT reagent was added into the scaffold and incubated at 37 °C, 5% CO_2, for 4 h. The media of the tube was replenished. 100 μl of dimethyl sulfoxide (DMSO) was added, and the scaffold tube was shaken for 20 min in the dark. The solution in each tube was transferred to each well of 96-well discs. Absorbance readings at 595 nm were recorded using a spectrophotometer (DTX 880, Beckman Coulter, Brea, CA, USA) at 24, 48, 72, 96, 120 and 144 h. The assay included blank wells containing culture medium only and negative control wells containing unseeded scaffold. The results were analysed by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

2.6 In Vitro Cartilage Formation

BMMSCs were cultured in chondrogenesis medium after 7 days of seeding on scaffolds. The medium was refreshed every 5 days. The scaffolds were assessed by histological staining with Alcian Blue reagent for chondrogenesis at day 3, 7, 21 and 28 after differentiation induction.

2.7 Scanning Electron Microscopy (SEM)

Differentiated cell-seeded scaffolds, BMMSC-seeded scaffolds and unseeded scaffolds were fixed in 4% paraformaldehyde and examined by a scanning electron microscope (Hitachi, Japan).

2.8 Gene Expression Evaluation

The specific markers of MSCs were assessed for the candidate BMMSCs. The cells of the third and fifth passages were used in these experiments. Total RNA from BMMSCs was extracted using Easy-Spin™ (iNtRON Biotechnology, South Korea) and according to the manufacturer's instructions. Target DNAs were amplificated by RT-PCR one-step kit (PCR Biosystems, UK) using gene-specific primers such as CD14, CD34, CD45, CD44, CD73, CD90, CD105, CD106 and *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)* (Table 1). The amplification process includes 15-min reverse transcription at 45 °C, 2-min polymerase activation at 95 °C, 40 cycles of amplification for all genes with 5-s denaturation at 95 °C and 15-s annealing at 55 °C (for *CD90*, *CD45*), 56.2 °C (*CD14*, *CD34*), 57.2 °C (*Gapdh*, *CD44*), 58.3 °C (*CD73*, *CD105*) and 59 °C (*CD106*). The amplification products were observed using electrophoresis method on 1.5% agarose gel and staining with ethidium bromide (Sigma, USA).

The chondrogenic profile gene expression was evaluated during chondrogenesis. Total RNA from chondrogenic-induced BMMSCs was extracted using Easy-Spin™ (iNtRON Biotechnology, South Korea), according to the manufacturer's instructions. DNA amplification was conducted by qRT-PCR one-step kit (PCR Biosystems, UK) using gene-specific primers, including *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)*, *collagen type I (col I)*, *collagen type II (col II)*, *collagen type X (col X)*, *sox-9*, *runx2* and *aggrecan* (Table 1). The PCR amplification process includes 15-min reverse transcription at 45 °C, 2-min polymerase activation at 95 °C, 40 cycles of amplification for all genes with 5-s denaturation at 95 °C and 15-s annealing at 56 °C (for *sox9*, *runx2*), 56.6 °C (*col2*), 57.2 °C (*Gapdh*, *col I*), 58.3 °C (*col X*) and 59 °C (*aggrecan*). The melting curves were carried out after that.

2.9 Statistical Analysis

All results were analysed by GraphPad Prism 6.0 software. P-value <0.05 was considered to be statistically significant (different).

3 Results

3.1 Polycaprolactone Scaffold Preparation

The scaffold size was designed to be $30 \times 30 \times 1$ (mm^3). Scaffold pore size was defined as 150–400 μm and the filament diameter as 200 μm (Fig. 1). After treatment with alcohol, PCL scaffolds were sterilized; the sterilized PCL scaffolds were ready for use in the next experiments.

3.2 Successful Isolation of Rabbit Bone Marrow-Derived Mesenchymal Stem Cells

After 3 days of culture, the cells adhered on flask surface and grew rapidly (Fig. 2a). Cell density reached 70–80% confluency after day 7. From the third passage, the morphology of the BMMSC population became homogenous

Table 1 Primer sequences for BMMSCs and chondrocytes

Gene	Primer sequences (5′-3′)		Product length (bp)	GenBank no.
Gapdh	Forward	AGACACGATGGTGAAGGTCG	166	NM_001082253.1
	Reverse	CTTGCCGTGGGTGGAATCAT		
CD14	Forward	TGCCTAAGGGACTGCCTG	132	NM_001082195.2
	Reverse	CAGGGACCAGGAACGGATT		
CD34	Forward	CATCCTGGGCACTACTGGC	115	XM_008268472.2
	Reverse	GCATGTGCAGACTCCTTTCC		
CD44	Forward	AGGTTTGGTGGAAGACCTGG	162	XM_002709048.3
	Reverse	CTTCCTCCTCTGCCATGAGT		
CD45	Forward	CAATTACCTGGACACCTCCTC	221	XM_008268698.2
	Reverse	CTGACAGCTTGAAGCACTTC		
CD73	Forward	GAGCTCACGATCCTGCACAC	142	XM_017345272.1
	Reverse	CTTGGCGGATCTGTTGCACT		
CD90	Forward	CTCTGTGCTCAGAGACAAGC	135	XM_002722718.3
	Reverse	CCAACCAGTCACAGGGAAAG		
CD105	Forward	CGCTCTGGTGCATCTACTCG	108	XM_008251029.2
	Reverse	CGATGCTGTGGTTCGTGCT		
CD106	Forward	TCCCCGAATCCAGATCTCTTGC	134	NM_001082152.1
	Reverse	CTCGCTCCTCACCTTCCCAT		
Sox-9	Forward	AGCTCACCAGACCTTGAGAC	197	XM_008271763.2
	Reverse	GTTGGGTACCAGTTGCCTTC		
Col I	Forward	CAATGGTGGCACCCAGTTTG	258	NM_001195668.1
	Reverse	GTGCAGCCATCGACAAGAAC		
Col II	Forward	GGCTGGAGGATTTGACGAGA	135	NM_001195671.1
	Reverse	CCAGGGTTGCCTTGAAATCC		
Col X	Forward	GTCCTTCTGGACCACCAGGA	100	XM_002714724.3
	Reverse	GGCTTCCCAGTGGCTGATAG		
Aggrecan	Forward	TGGGTGTCAGGACCGTGTAC	155	XM_008251723.2
	Reverse	CGTCTGGACCGTGATGTCCT		
Runx2	Forward	GATGACACTGCCACTTCTGAC	165	XM_008262992.2

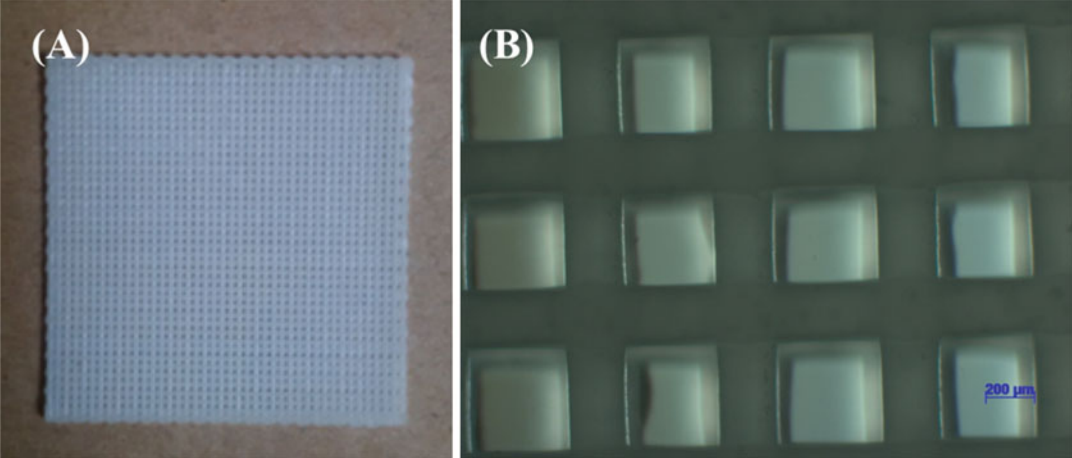

Fig. 1 A typical PCL scaffold produced by IAMI2. (**a**) The size of the scaffold was determined as 30 × 30 × 1 mm^3. (**b**) The pore size was 150–400 µm in diameter

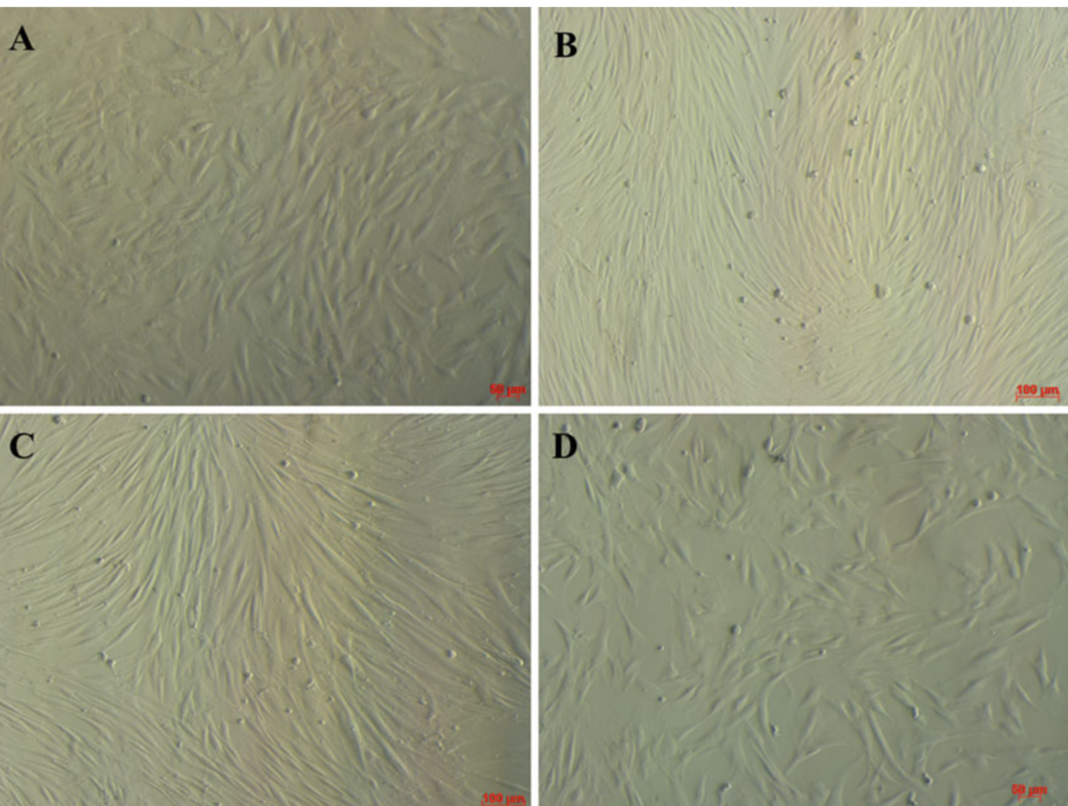

Fig. 2 The morphology of BMMSCs. The heterogeneous population of adherent cells in passage 1 (**a**); the cell population became more homogenous in passage 3 (**b**), passage 5 (**c**) and passage 7 (**d**)

(Fig. 2b), and this was maintained until passage 7 (Fig. 2c, d).

The candidate cells were successfully induced to differentiate into chondrocytes, adipocytes and osteocytes. Indeed, 8 days after culturing in adipogenic inducible medium, the intracellular lipid droplets appeared. These droplets could be stained with Oil red O (Fig. 3a). After 21 days of culture in osteogenic medium, BMMSCs could deposit calcium which could be stained by Alizarin Red S staining (Fig. 3b). After 28 days of chondrogenic induction, BMMSCs also successfully deposited proteoglycan that was visualized by Alcian Blue staining (Fig. 3c). However, BMMSCs in control (placebo) medium could not form oil droplet nor deposit calcium or proteoglycan (Fig. 3d–f).

The expression of particular markers of MSCs was confirmed by RT-PCR. The markers were as follows: CD14, CD34, CD44, CD45, CD73, CD90, CD105 and CD106. The results are presented in Fig. 4. BMMSCs expressed CD14 and CD45 at passage 3; however, they did not express them at passage 5. At the latter passage number, BMMSCs expressed CD44, CD73, CD105 and CD106, while they were negative for CD14, CD34, CD45 and CD90 (Fig. 4).

3.3 BMMSCs Were Successfully Transfected with GFP

After 24 h of lentivirus infection, the percentage of GFP-positive cells reached 64.31%, as evaluated by flow cytometry (Fig. 5a, d, g, j)). After 2 days of selection with puromycin (2 μg/ml), the percentage of GFP expressing BMMSCs increased to 92.93% (Fig. 5b, e, h, k); after 7 days of selection under the same conditions, the percentage reached 99.98% (Fig. 5c, f, i, l).

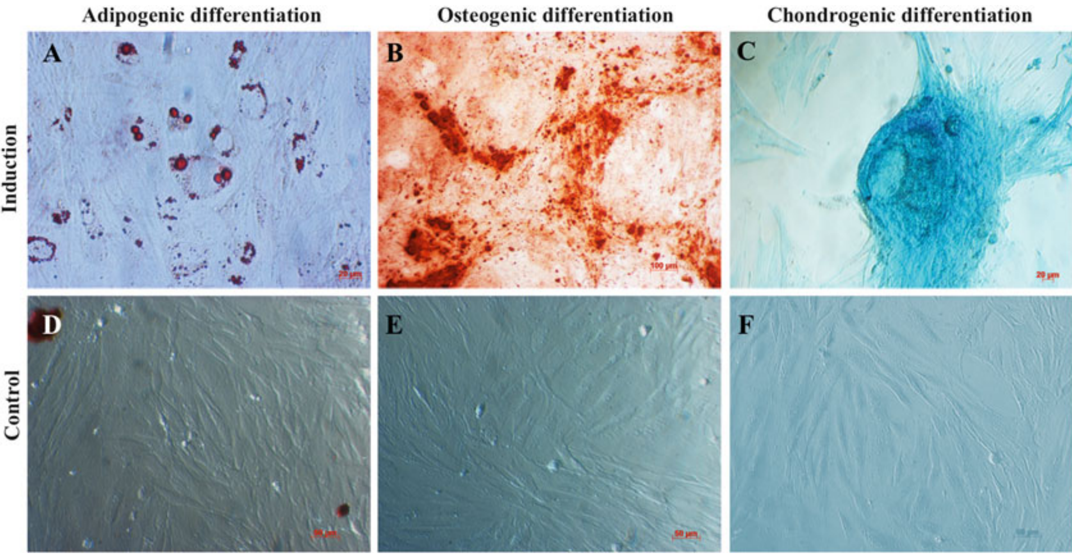

Fig. 3 The differentiation ability of candidate BMMSCs. Lipid accumulation (**a**), calcium deposition (**b**) and proteoglycan accumulation(**c**) on BMMSCs were visualized by Oil Red (**a**), Alizarin Red (**b**) and Alcian Blue (**c**) staining, respectively. The cells could differentiate into adipocytes, osteocytes and chondrocytes. However, for the control (placebo) groups, BMMSCs were negative for these stainings (**d**, **e**, **f**)

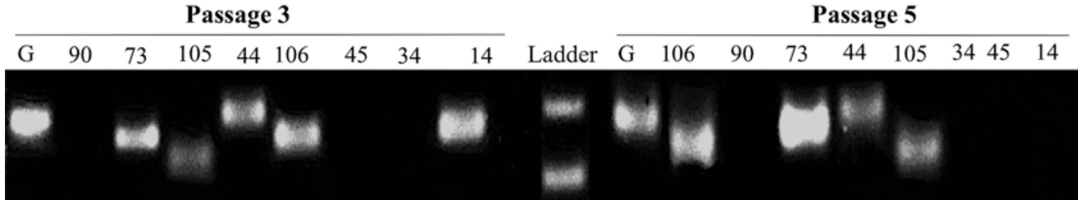

Fig. 4 Gene expression of MSC-specific markers. BMMSCs at passages 3 and 5 were positive for CD73, CD105, CD44 and CD106, but negative for CD90, CD45 and CD14. The absence of CD34 was observed at passage 5 as opposed to passage 3. G, GAPDH; 90, CD90; 73, CD73; 105, CD105; 44, CD44; 106, CD106; 45, CD45; 34, CD34; 14, CD14

3.4 BMMSCs Could Adhere and Proliferate on PCL Scaffolds and Collagen-Coated PCL Scaffolds

The results showed that BMMSCs could attach on surfaces of both PCL and PCL/col scaffolds. These cells were evenly distributed throughout the PCL/col scaffold. However, under the microscope, cell density was higher on PCL/col scaffolds than on PCL scaffolds (Fig. 6a).

The results of the MTT assay supported this observation. From 24 to 48 h, the rate of proliferation was quite slow, but this rate rapidly increased from 48 to 72 h. The growth reached a stable phase after 48 h and a dead phase after 120 h of seeding (Fig. 6b). Besides, the OD values indicated that the number of BMMSCs attached on PCL/col scaffolds was greater than on PCL scaffolds. These results demonstrate that adhesion of BMMSCs was enhanced on PCL/col scaffolds versus PCL scaffolds. Therefore, PCL/col scaffolds were used in the next experiments.

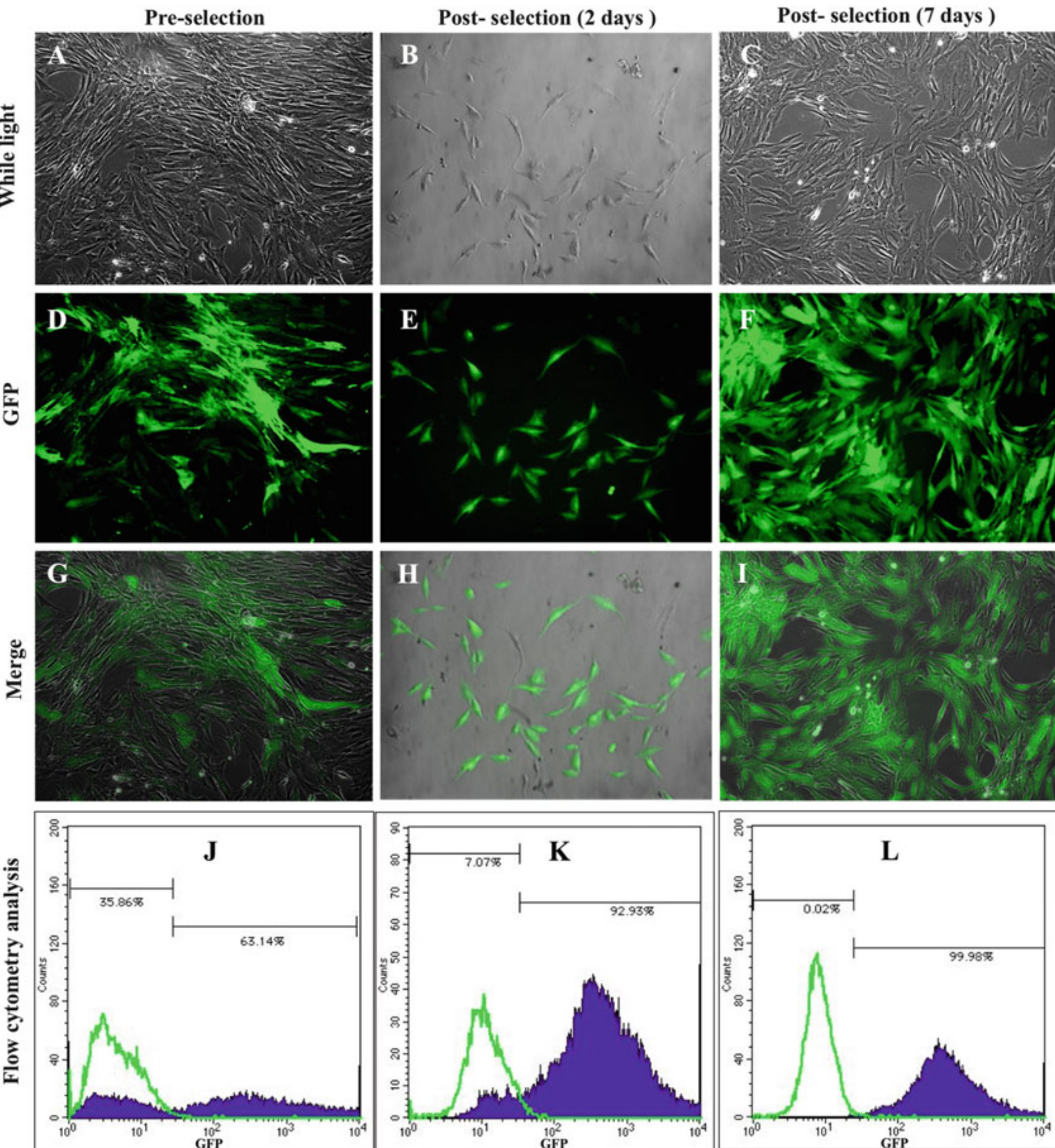

Fig. 5 GFP gene transfection of BMMSCs. The percentage of GFP-positive cells significantly increased after culture in selection medium in 2 days (**b**, **e**, **h**, **k**) and 7 days (**c**, **f**, **i**, **l**) compared to before selection (**a**, **d**, **g**, **j**)

3.5 BMMSCs Were Differentiated into Chondrocytes in the Scaffold

On the 7th day post induction, the BMMSCs continued to expand in the pores of the scaffold (Fig. 7b, c). At the 14th day, these cells began aggregating with each other (Fig. 7d). The condensation continued until the 35th day (Fig. 7f). As a result, the morphological changes of BMMSCs were recorded after induction.

During induction, the proteoglycan deposit of BMMSCs was clearly observed from the 7th day. The proteins were detected via Alcian Blue reagent; chondrogenic-induced BMMSCs were stained as blue. At the 3rd and 7th days, the proteoglycan expression showed as dim blue (Fig. 8b, c). However, deep-blue staining was

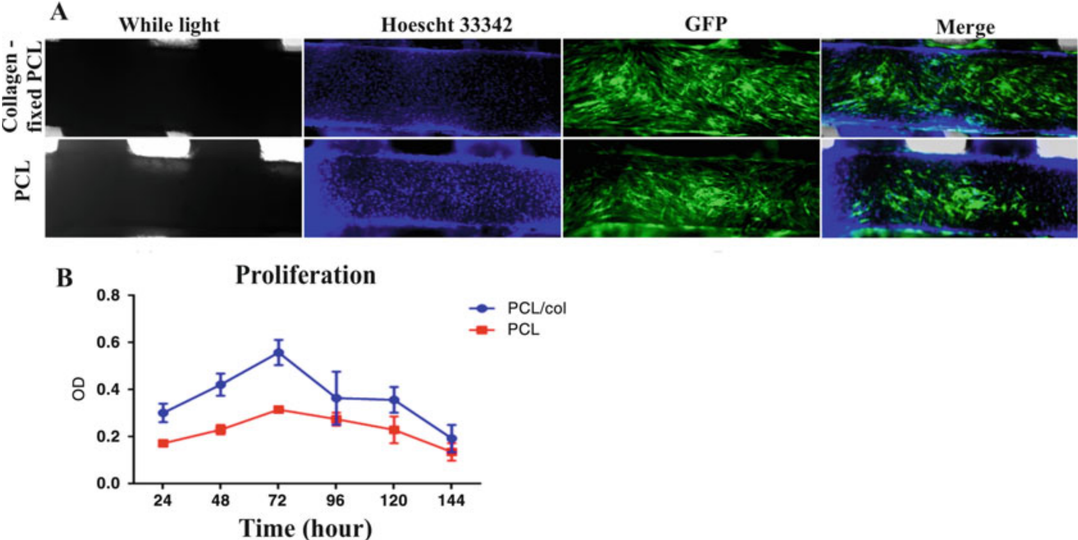

Fig. 6 The adherence and proliferation of rBMMSCs seeded on PCL/col versus PCL scaffolds. (**a**) BMMSCs were able to attach on both PCL/col and PCL scaffolds. (**b**) The proliferation of adherent cells on PCL/col scaffold was stronger than on PCL scaffold

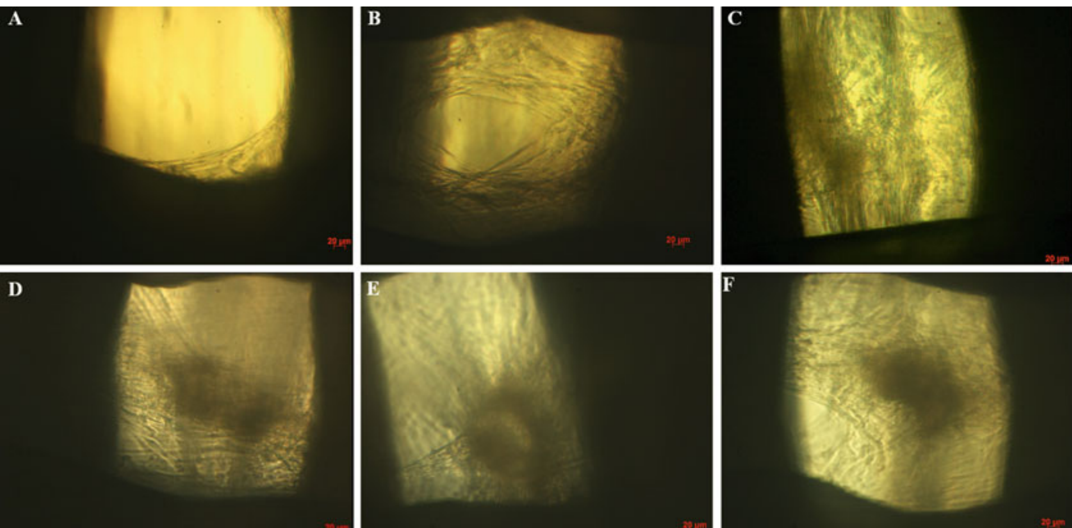

Fig. 7 The morphological changes of BMMSCs after chondrogenic induction. (**a**) BMMSCs seeded on PCL/col scaffold before induction; (**b**) BMMSCs expanded in pores of scaffold on the 7th day; (**c**–**f**) BMMSCs aggregated into pellet-like aggregation at days 14, 21, 28 and 35 after induction

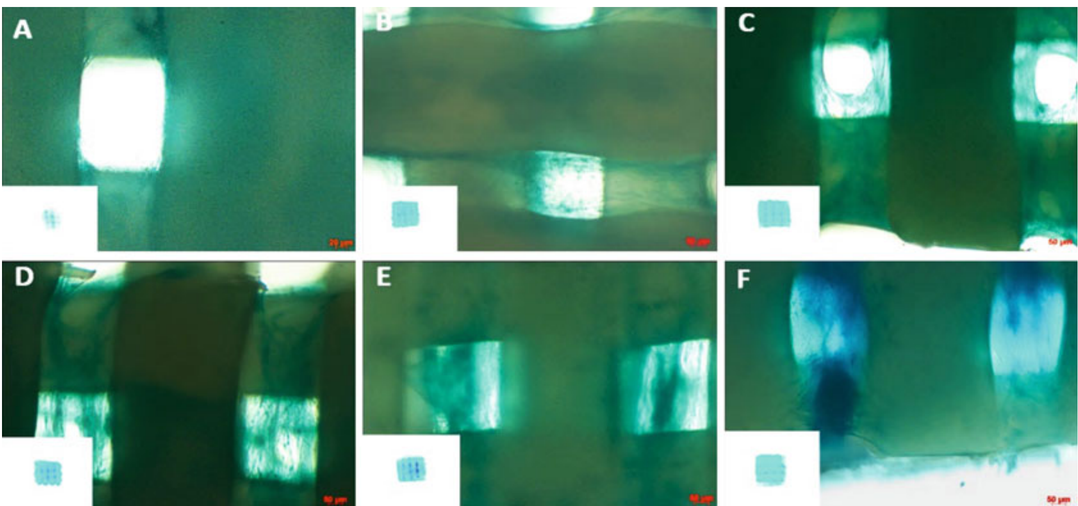

Fig. 8 The proteoglycan accumulation of BMMSCs on PCL/col scaffold before induction (**a**) and after 3 (**b**), 7 (**c**), 14 (**d**), 21 (**e**) and 28 (**f**) days of induction as assessed by Alcian Blue staining

observed at the 14th, 21th, 28th and 35th days of induction (Fig. 8d, e, f). These results demonstrate that proteoglycan proteins are produced by BMMSCs during the chondrogenesis process.

The synthesis of ECM proteins caused some changes to the scaffold surface which were detectable by SEM. The results from SEM showed that there were differences in the surface of scaffold and shape of cells between undifferentiated BMMSC scaffold and differentiated BMMSC scaffold (Fig. 9). An appearance of collagen-like composition was seen in the differentiated BMMSC samples (Fig. 9b) but not in the nondifferentiated samples (Fig. 9c). The shape of normal BMMSCs was different from that of differentiated BMMSCs on PCL/col scaffolds. These results show that BMMSCs are able to manufacture collagen-like extracellular matrix proteins after chondrogenesis induction on PCL/col scaffolds.

During the chondrogenic induction period of BMMSCs on the scaffold, gene expression was evaluated for the BMMSCs; the genes assessed were *sox9*, *col2a1*, *col1a2*, *aggrecan (acan)*, *runx2* and *colX*. Compared to the placebo group, in the chondrogenic-induced group, the BMMSCs showed altered gene expression (towards chondroblasts) after induction for 3, 7, 14, 21 and 28 days (Fig. 10). Sox9 expression gradually increased over time. At the 21st day, the expression of Sox9 significantly increased compared to the previous days and compared to the control group ($p < 0.05$). At the 28th day, sox9 expression significantly declined compared to the 21st day, but it was still higher than the control group ($p < 0.05$). Col2a1 expression was observed in normal BMMSCs. However, it was also seen in chondrogenic-induced BMMSCs at the 7th and 14th day of induction; compared to BMMSCs, its expression in chondrogenic-induced BMMSCs was significantly higher, reaching 10.02 ± 3.28- and 21.97 ± 2.72-fold greater after the 7th day and 14th day, respectively. However, this expression considerably decreased after the 21st day of induction to lower than normal BMMSCs, thinking the decrease was not significant ($p < 0.05$). One of the early expressed genes of chondrogenesis, col1a2, was strongly expressed at the 7th day and declined after that. Finally, the expression level of aggrecan (one of the proteoglycans) was highest compared to other genes, especially at the 7th day post induction.

Moreover, mature chondrocytes were evaluated for expression of genes such as *runx2* and *colX*. The results showed that *runx2* expression was highest and considerably higher than that of the control group at day 7, reaching

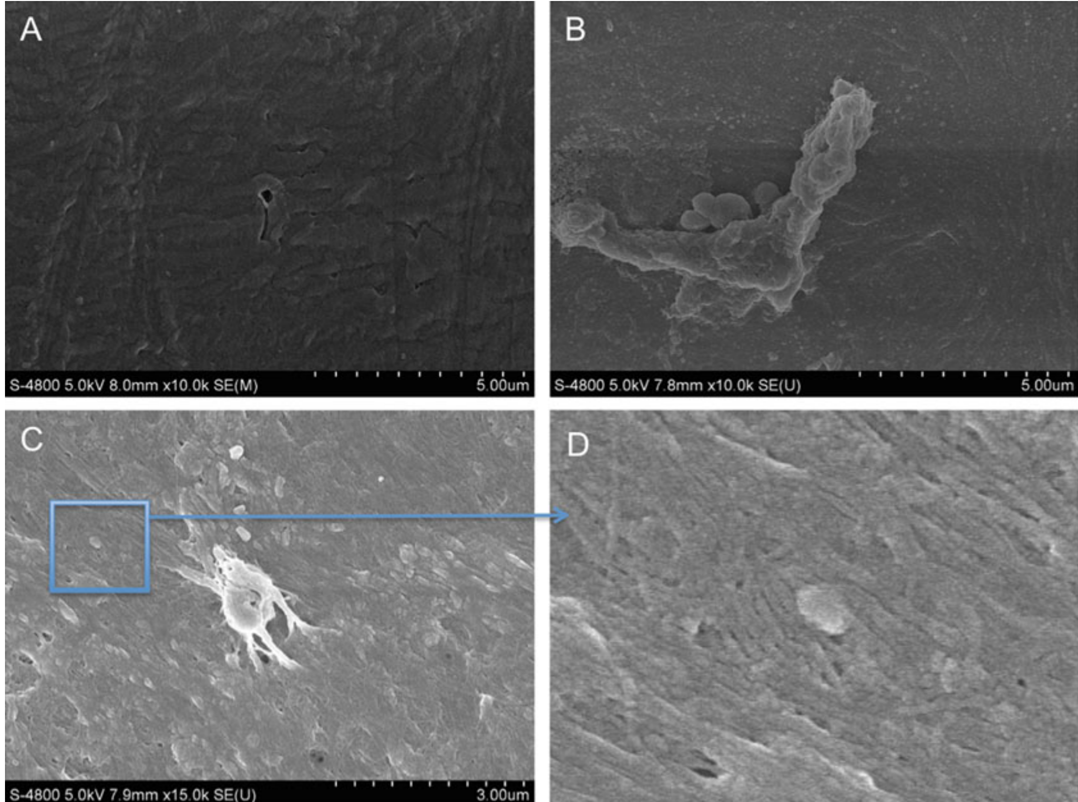

Fig. 9 The growth and differentiation of BMMSCs on PCL/col. SEM capture of unseeded PCL/col scaffold (**a**), BMMSC seeded PCL/col scaffold after 7 days (**b**) and BMMSC seeded PCL/col scaffold after chondrogenesis induction for 21 days (**c**). Collagen-like composition was observed in the differentiated rBMMSCs after 21 days (**d**)

39.40±19.15-fold greater. After that time, its expression significantly decreased. As for *colX*, it was expressed from the 7th day of induction, reaching 22.92±5.00-fold greater than the control; its expression was highest around the 21st day of induction (Fig. 11).

Thus, the results demonstrated that BMMSCs were able to express genes for chondrogenesis, such as *col2a1*, *col1a2*, *aggrecan* and *sox9*. Moreover, they also expressed genes related to mature chondrocytes, such as *runx2* and *colX*. When cultured in chondrogenic-induced medium, all of the above-mentioned genes were altered in their expression level.

4 Discussion

The microenvironment is impacted by the scaffold, culture medium and inducible factors, and all play an important role in the growth of stem cells. The microenvironment can affect the migration, proliferation, morphogenesis and differentiation of stem cells (Ayala et al. 2011). Many elements of the microenvironment, such as the chemical properties and composition of scaffold, strongly impact both the behaviour and differentiation of cells (Elisseeff et al. 2006; Hwang et al. 2008). For example, the combination of human MSCs and polyacrylamide

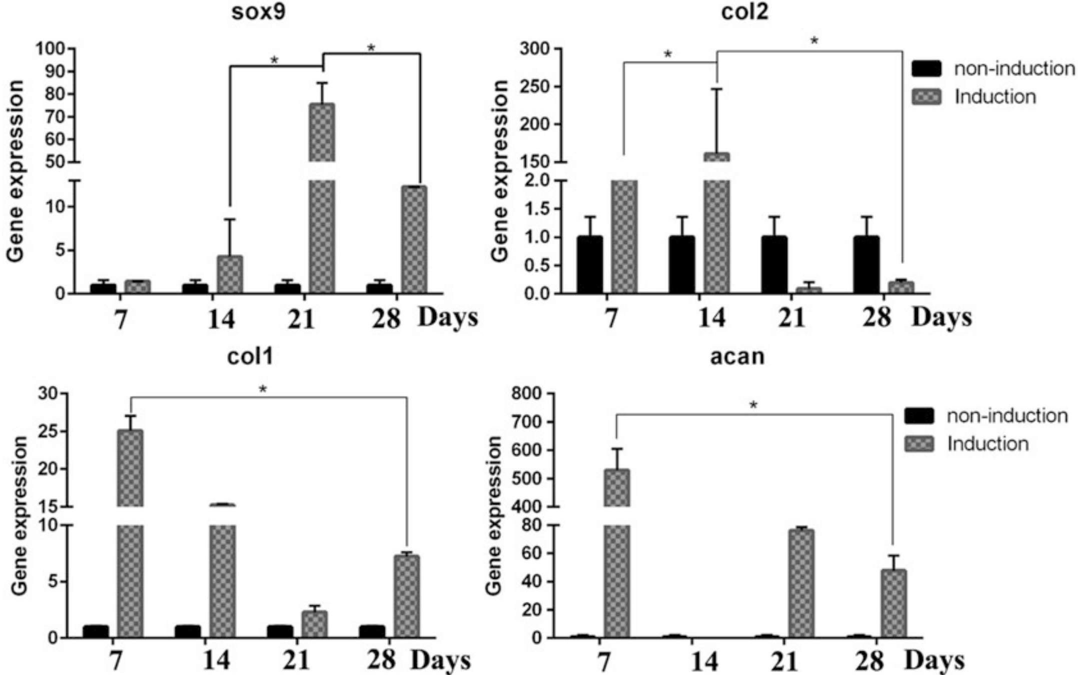

Fig. 10 The chondrogenic gene expression of BMMSCs after induction for 7, 21, 14 and 28 days. Sox9 expression increased over time, while col2a1, col1a2 and acan (aggrecan) expression peaked on the 7th and 14th days but decreased after that (*p < 0.05; n = 3)

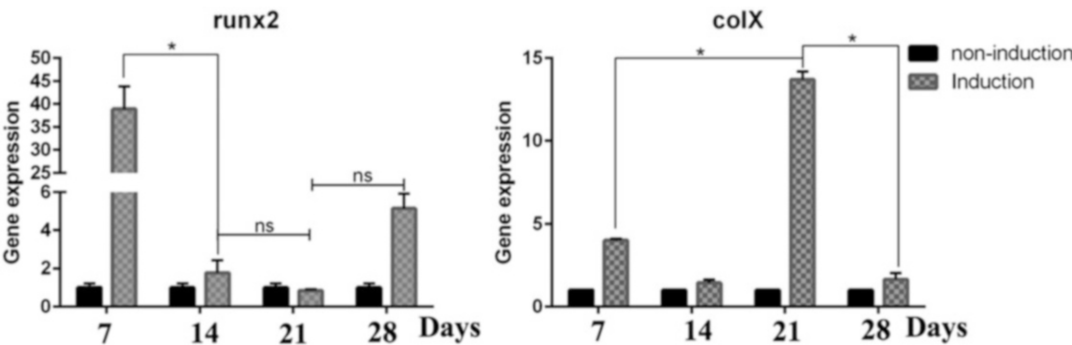

Fig. 11 Differentiated BMMSCs express mature chondrocyte-related genes on PCL/col scaffold (*p < 0.05; n = 3)

hydrogel was shown to be suitable for neuronal regeneration, while the coordination of human MSCs and collagen or polycaprolactone was suitable for skeletal differentiation (Krishna et al. 2016).

Scaffolds used for tissue regeneration are comprised of both natural and synthetic materials. Natural materials are the polymeric proteins of native extracellular matrix; these include fibrinogen, collagen, chitosan, hyaluronic acid, etc. They can enhance the adherence of cells via attachment of cell surface receptors and can control cell function by supplying a physical microenvironment (Dawson et al. 2008). Meanwhile, synthetic materials could be fabricated with advantageous properties for regenerative medicine, such as biodegradability, biocompatibility and safety.

Previous studies have demonstrated that the combination of synthetic and natural material scaffolds was more efficient for the adhesion and differentiation of cells compared with utilizing only synthetic polymer. Hwang et al. (2011) showed that MSCs could better attach on collagen-treated polyethylene glycol (PEG) scaffolds than non-treated scaffolds or collagen-treated scaffolds (Hwang et al. 2011). In our study, however, we evaluated adhesion of BMMSCs on collagen-treated PCL versus PCL-only scaffolds. The results showed that collagen-treated PCL scaffolds enhanced the adhesion of BMMSCs compared to PCL-only scaffolds.

After seeding BMMSC on the scaffolds, the cells were induced to differentiate into chondrocytes. In the chondrogenesis process, MSCs undergo many different stages. In each stage, the cells undergo different morphological changes. In our study, the changes were recorded, while BMMSCSs were induced into chondrocytes. At the 7th day of induction, BMMSCs migrated into the scaffold pores. The cells were in the condensation stage of chondrogenesis. Indeed, by cell-cell and cell-matrix interactions, hyaluronidase activity increased to reduce hyaluronan, which prevented cell-cell interaction in extracellular matrix (Gao et al. 2014). Therefore, the cell-cell interactions were close.

During that time, cell adhesion molecules (such as N-cadherin and N-CAM) were expressed. Fibronectin also appeared in the ECM to regulate N-CAM, while collagen type I was continually expressed (Hidaka and Goldring 2008; Oberlender and Tuan 1994). The expression of *collagen type I* was regulated by *runx2* gene. Thus, BMMSCs tended to contact with each other and aggregate inside the pores of scaffold after induction. Moreover, our results demonstrated that *runx2* was highly expressed, leading to the upregulation of *collagen type I* at the 7th day of induction.

During the condensation stage, the cells aggregated into an area which had high cell density and enhanced proliferation. ECM was produced more and ECM composition changed. In the initial stage, the transcription factors *sox9*, *sox5* and *sox6* were expressed to regulate the expression of structural proteins, including collagen type II and aggrecan. Sox9 is known to be a marker of early chondrogenesis and is required for the expression of cartilage-specific matrix protein by binding to the enhancer region (Lefebvre et al. 1997). However, the expression of these genes was inhibited in prehypertrophic chondrocytes. In the prehypertrophic stage, *collagen type X* expression was upregulated in cells. In previous research studies, collagen type X was shown to be released from BMMSCs into culture supernatant after induction for 21 days (Grassel and Ahmed 2007). The release was promoted by transcription factor *runx2*. Indeed, *runx2* and *collagen type X* are seen as marker genes for hypertrophic chondrocytes (Kozhemyakina et al. 2015). Many studies have found that upregulation of the *sox9* gene can inhibit differentiation into hypertrophic chondrocytes and the maintenance of the prehypertrophic process (Okazaki and Sandell 2004).

In our study, the RT-qPCR results showed that expression of *collagen type II* and *aggrecan* was highest at the 7th day and 14th day of induction, respectively. The expression of *sox9* increased at the same time. This shows that BMMSCs have undergone the initial step to become chondroblasts. At the 21st day of induction, expression of these genes was decreased, while *collagen X* and *sox9* expression were upregulated compare with the other days. The expression of *collagen type X* demonstrated that the cells had the ability to differentiate into prehypertrophic chondrocytes. On the other hand, the upregulation of *sox9* indicated that the prehypertrophic stage was maintained at this time. The expression of *sox9* significantly declined at the 28th day, and the cells tended to differentiate into hypertrophic chondrocytes. The results demonstrate that BMMSCs were induced to differentiate into immature chondrocytes in the first 7 days and that the differentiation proceeded to terminal differentiation (mature chondrocytes) at day 28 of induction.

5 Conclusion

This study showed that BMMSCs can attach and proliferate on both PCL/col and PCL scaffolds. The adherent efficacy of BMMSCs on PCL/col scaffolds was significantly better than on PCL scaffolds. BMMSCs can successfully be differentiated into mature chondrocytes. Indeed, BMMSCs changed their shape on PLC/col scaffolds, and there was accumulation of proteoglycans. The scaffold surfaces were also modified with collagen-like fibre structures. Differentiated BMMSCs expressed chondrogenesis-associated genes after 7, 14, 21 and 28 days of induction, especially the gene collagen type II, aggrecan and collagen type X. Our study results suggest that in vitro cartilage can be produced by seeding BMMSCs on collagen-coated PCL scaffolds with chondrogenesis-inducing medium.

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Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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Stem Cell Therapy for Tendon Regeneration: Current Status and Future Directions

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Florian Geburek, and Thomas Skutella

Abstract

In adults the healing tendon generates fibrovascular scar tissue and recovers never histologically, mechanically, and functionally which leads to chronic and to degenerative diseases. In this review, the processes and mechanisms of tendon development and fetal regeneration in comparison to adult defect repair and degeneration are discussed in relation to regenerative therapeutic options. We focused on the application of stem cells, growth factors, transcription factors, and gene therapy in tendon injury therapies in order to intervene the scarring process and to induce functional regeneration of the lesioned tissue. Outlines for future therapeutic approaches for tendon injuries will be provided.

Keywords

Tendon lesion · Stem cells · Growth factors · Transcription factors · Regenerative therapy

Abbreviations

AD-MSC	Adipose-derived mesenchymal stem cell
ADNC	Adipose-derived nucleated cell
ASC	Adipose stromal cell
ASO	Antisense oligonucleotide
AT-MSC	Adipose tissue-derived mesenchymal stem cell
BGN	Biglycan
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMP	Bone morphogenetic protein
cPLA2	Cytosolic phospholipase A2
COL4A1	Collagen type IV alpha 1
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
CRISPR	utilized clustered regularly interspaced short palindromic repeats
CTGF	Connective tissue growth factor
DCN	Decorin
ECM	Extracellular matrix

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EGR	Early growth response protein
EGR-1	Early growth response protein 1
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FCER1g	Fc fragment of IgE receptor Ig
FGF	Fibroblast growth factor
FMOD	Fibromodulin
FN	Fibronectin
GDF	Growth and differentiation factor
GF	Growth factor
GFP	Green fluorescent protein
GH	Growth hormone
hASC	Human adipose-derived stem cell
HP	Hydroxylysylpyridinoline
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IGF	Insulin-like growth factor
IL	Interleukin
iPSC	Induced pluripotent stem cell
IRAP	Interleukin-1-receptor-antagonist protein processing system
LOXL4	Lysyl oxidase like 4
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MCP	Mast cell protease
MKX	Mohawk
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MRL	Murphy Roths Large (mouse)
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PRP	Platelet-rich plasma
RUNX-2	Runt-related transcription factor 2
SCX	Scleraxis
SDFT	Superficial digital flexor tendon
SMA	Smooth muscle actin
SOX	SRY-box gene
TDC	Tendon-derived cell
TDGFß1	Transforming growth factor ß1
TGF	Transforming growth factor
THBS-2	Thrombospondin-2
TN-C	Tenascin-C
TNFα	Tumor necrosis factor-α
TNMD	Tenomodulin
TOF	Time of flight
TPC	Tendon precursor cell
TSC	Tendon stem cell
TSPC	Tendon stem/progenitor cell
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

1 Introduction

Some of the most frequent orthopedic diagnoses are tendon injuries, which often present clinical physicians with a challenge, mainly due to the inadequate response to treatment and the need for extended rehabilitation phases. Sutures, autografts, allografts, and synthetic prostheses have so far been administered in therapy for the recovery of injured tendons. These approaches previously failed to accomplish a continuing solution, as affected tendons never fully recovered concerning stability and functionality. Tendon lesions are not only intense; they often manifest in chronic and degenerative diseases. In adults the healing tendon generates fibrovascular scar tissue and thus never recovers to the former qualities, neither histologically, mechanically, nor functionally.

The exact processes and mechanisms of tendon development and fetal regeneration in comparison to adult defect repair and degeneration remain only partially understood. Since the adult tendon cannot regenerate itself after injury, researchers and physicians lately became more focused on the concept of scarless tendon tissue regeneration by the inclusion of growth factors (GFs) and cell therapeutics in combination with conventional medical interventions.

Tendons consist of highly specialized parallel-oriented collagenous fibers embedded in endotenon, epitenon, paratenon, and tendon sheaths. They join muscles to bones and thus effectively convey mechanical forces (Birk and Trelstad 1986; Woo et al. 2007; Connizzo et al. 2013). Both surgical and nonsurgical methods of treatment for tendon injuries result partially in

satisfying outcomes (Rosso et al. 2015; Rahr-Wagner et al. 2014). Nevertheless, the recovered tendon tissues frequently appear histologically unorganized and with less inferior mechanical strength, eventually leading to recurrence (Haggmark et al. 1986; Thomopoulos et al. 2002). Furthermore, instability due to partial healing remains a risk factor for relapses or the development of osteoarthritis as aftereffect (Friel and Chu 2013; Frank et al. 1995).

Understanding tendon tissue and cellular repair mechanisms in more detail and utilizing modern molecular and cellular tools could help to finally make the breakthrough in finding treatment approaches especially for tendon conditions and thereby improve disease management programs. Recently, a thorough quantitative proteomic analysis of the human tendon including insoluble proteins was documented (Sato et al. 2016), which provides target criteria for stem cell- or tissue-engineered tendon.

Since tendon cell differentiation pathways can be better understood, specific regulatory factors for the tenocyte phenotype, including tenocytes and cells of the endotenon, epitenon, paratenon, and tendon sheath, are capable of leading to advanced cell therapy and pharmacological treatment methods for improved tendon healing or tendon tissue engineering. Biologic substances including stem cells, GFs, small molecules mimicking transcription factor pathway induction, and natural biomaterials, together or separately, are options to be administered to the lesion site in the different stages of wound healing in order to inhibit the generation of scar tissue.

The ability of fetal tendon tissue to regenerate was examined and contrasted to adult tendon scarring after lesion by Fayata et al. (2006), who detected that injured adult tendons display higher transforming growth factor β1 (TGFβ-1), fibroblast growth factor (FGF)-2, and CD44 levels at the lesion site, while the fetal tendon tissue showed almost no alterations (Martin et al. 2003a, b). This combination of factors might block a proliferation and tissue-specific tendon regeneration. At the "New Frontiers in Tendon Research meeting" (Mt. Sinai Medical Center, NYC, September 2014), several reviews disclosed the utilization of Murphy Roths Large (MRL) mice in various laboratories in order to analyze advanced tendon regeneration. In about 80% of the cases, they reported restored tendon functionality (Bell et al. 2015). MRL inflammatory cell types were analyzed and displayed a higher amount of macrophages, neutrophils, and mast cells in the lesion site after the injury. In the MRL mouse, myeloperoxidase (MPO) and mast cell protease 7 (MCP7)/tryptase alpha molecules were highly upregulated. Huang et al. also reported MCP7 as a potent inflammatory mediator, which is pro-angiogenic and influences formation and fibrinogen/integrin interactions (Huang et al. 1997). Moreover, in the MRL multiple molecules, like connective tissue growth factor (CTGF) and lysyl oxidase like (LOXL4), which also function in scar generation, are limited. The mass cell high-affinity Ig receptor, Fc fragment of IgE receptor Ig (FCER1g), plays a key role in inflammation and was found 57 times decreased in MRL. This fact should cause less inflammatory cell-mediated tissue destruction (Takaya et al. 2005).

According to Howell et al. (2017), neonate mouse tendons can completely recover to former strength by recruitment of scleraxis-(SCX) positive tendon stem cells (TSCs), whereas in contrast adult tendons recover with a remaining fibrovascular scar. In adults extensive proliferating α smooth muscle actin (SMA)-expressing cells induce a lasting scar formation, since tenogenic SCX-regenerating cells do not proliferate.

This raises the question which therapeutic options are available for adult organisms that could replace simple scar forming repair mechanisms after tendon lesions by tissue regeneration.

In this review, we first describe the different stages of tendon healing in the context of tendon biology and therapeutic alternatives. Then, this report explains and evaluates in more detail actual biological treatment options including stem cells and growth and transcription factors for recovering injured tendons with functional tissue-specific regeneration.

1.1 Tendon Injuries and Healing

The area of tendon repair is a complicated dynamic environment, which consists of various types of cells and tissue structure with its highly specified extracellular matrix (ECM). It is a reason for discussion what factors contribute to intrinsic and extrinsic tendon healing and how extra-tendinous cells are involved in the process.

Commonly, the recovery of injured or compromised tendons is accomplished in three stages, including specific cellular and molecular cascades. These stages may coincide as their continuation correlates with location and severity of the injury (Lin et al. 2004; Voleti et al. 2012; Yang et al. 2013). After tendon injury, the three partially coinciding levels of healing include the inflammation, proliferative, and remodeling stages (Hope and Saxby 2007).

During the first stage, the inflammatory stage, after the tendon fibers and barriers have been disrupted, tenocytes, endotenon, and cells of the endotenon, epitenon, paratenon, and tendon sheath react to the cell damage. Immediately after injury, the acute inflammatory response is initiated resulting in recruitment of circulating inflammatory cells including macrophages. During this stage, hemostasis begins to form shortly after the injury, and afterward pro-inflammatory cytokines guide red blood cells, leukocytes, and platelets equipped with specific GFs, and endothelial chemoattractants pass through the lesion (Lin et al. 2004). Hemostasis is achieved by endothelial-activated vasoconstriction and the clotting cascade. Platelets degranulate alpha granules, leading to secretion of GFs and pro-inflammatory cytokines. The building of a vascular network, which has the function to sustain the newly generated fibrous tissue at the injury site, is induced by secreted angiogenic factors. These first vascular reactions are vital, as it was demonstrated that a decrease of blood supply impedes the healing process, although these reactions are intense and disorganized (Fenwick et al. 2002). While a fibrin clot is generated causing transient stiffness, monocytes transforming to macrophages and neutrophils process the necrotic debris, and with lacking efficient numbers of tenogenic precursor cells, the adult tendon fibrotic repair is mediated by proliferating fibroblast cells that continue to form a permanent scar (Godwin et al. 2013, 2014). During the inflammatory stage, macrophages are a vital cellular component and thus possibly provide for future therapeutic measures. Macrophages can be separated into classically M1- or alternatively M2-activated cells. Inflammation and extracellular matrix deposition are caused by M1 macrophages, which also support the formation of scars. M2 macrophages, on the contrary, support the proliferation of cells, suppress inflammation, and thus might provide an option for regenerative tissue modeling.

In this context, the necessity for a tentative assessment of the administration of anti-inflammatory drugs in tendon therapy has been reported (Ferry et al. 2007; Zhang et al. 2015). Zhang et al. observed that interleukin (IL)-1β irreversibly inhibits tenogenic proliferation and differentiation and alters metabolism in injured tendon-derived progenitor cells in vitro. According to former studies on tendon lesions, the mRNA levels of inflammatory cytokines like IL-1β are extremely upregulated (Zhang et al. 2015). In mice, the wounded Achilles tendon was examined to find out the influence of IL-1β in the tendon healing process and of IL-1β on the tendon precursor cells (TPCs) in vitro. The tendon cell marker expression of SCX and tenomodulin (TNMD) was notably minimized after the administration of IL-1β, and the tendon-specific gene expression of Collagen I and III, biglycan (BGN), and fibromodulin (FMOD) in TPCs was found downregulated. Intriguingly, removing IL-1β did not influence the chondrogenic and osteogenic differentiation capability, whereas the tenogenic and adipogenic differentiation was not restored. These outcomes imply that the tenogenic potential is greatly and unalterably hindered by IL-1β. This implies that during the inflammatory stage of tendon healing, the normal function of local or transplanted tendon progenitor cells might profit from an IL-1β inhibition.

Experiments with regular anti-inflammatory drugs which affect selective and nonselective cyclooxygenase (COX) inhibitors imply that due to their adverse effect on Collagen production and healing strength, their administration during early tendon injury stages should be carefully considered (Connizzo et al. 2014; Ferry et al. 2007).

Furthermore, also the matrix can be destroyed by inflammatory cytokines such as IL-1β, which also results in a loss of biomechanical properties of the tendon. The successors are induced inflammatory mediators like cytosolic phospholipase A2 (cPLA2), COX-2 and COX-3, and prostaglandin E2 (PGE2) and the higher expression or activities of matrix metalloproteinases (MMPs) like MMP1, MMP3, and MMP13 in tenocytes (Archambault et al. 2002; Sun et al. 2008; Thampatty et al. 2007; Tsuzaki et al. 2003). It could be inferred that an ongoing MMP occurrence reinforces proliferative fibrotic tissue synthesis.

As demonstrated in a study by Zhang et al., the microenvironment of tendon progenitor niche could be modified in the healing tendons and thus hinder the normal recovery of its biomechanical properties. Zhang et al. conclude that for an effective stimulation of tendon regeneration, it is essential to observe specific time points for the control of inflammatory cytokine actions (Zhang et al. 2015).

During the second stage of tendon healing, the proliferative stage, recruited fibroblasts synthesize ECM components, especially associated with the synthesis of plentiful randomly organized ECM components, comprising proteoglycans and Collagens, mostly Collagen type III. This repair stage is denoted by intense synthetic activity and directed by M2 macrophages and fibroblasts. Macrophages, which first function phagocytic, become reparative a few days after the injury and release GFs and trigger cell recruitment (Leadbetter 1992; Massimino et al. 1997). An augmented cellularity and large amounts of absorbed water are a further characteristic of this stage. In the meantime, fibroblasts have overgrown the lesion site by scarring.

In this context, mesenchymal stem cells (MSCs) might possess therapeutic functions supporting wound healing and minimizing scar formation, which was reported in preclinical and clinical studies. MSCs might also be a promising therapeutic option for scar treatment. In goats, transplanted MSCs decreased scarring; in a human in vitro experiment, administering human MSC-conditioned medium or lysate to human keloid fibroblasts minimized fibroblast cell proliferation and migration (Fong et al. 2014; Liang et al. 2013). Fang et al. reported in 2016 that bone marrow-derived MSCs have the ability to inhibit the proliferative and pro-fibrotic phenotype of scarring through paracrine signaling (Fang et al. 2016). In human fibroblasts in vitro, the pro-fibrotic gene expression, like CTGF, plasminogen activator inhibitor (PAI-1), TGFß-1, and TGFß-2 were markedly diminished by MSC-conditioned medium. The anti-fibrotic gene expression on the opposite, like that of TGFß-3 and decorin (DCN), was significantly increased under equal culture conditions. In conclusion, Fang et al. found that the diminished Collagen I and fibronectin expression, and the low hydroxyproline levels in supernatant cell culture, suggested that ECM synthesis in fibroblasts was suppressed by the MSC-conditioned medium.

Then, 6–8 weeks post-injury, follows the third phase or remodeling stage, which consists of two substages and lasts around 1–2 years, relative to patient age and condition. A decline in cellularity and matrix production specifies the first substage, the consolidation, when tissue increases in fiber content by Collagen type I to Collagen type III replacement. Along the longitudinal axis of the tendon, collagen fibers begin to rearrange and thereby recondition tendon stiffness and tear resistance. The maturation stage begins about 10 weeks subsequently; it is characterized by an increase in collagen fibril cross-linking and the formation of more mature fibrotic tissue. Meanwhile, the ECM comes more into line, and cell density and overall synthetic activity become gradually less. Phase three extends to 1 year and longer. The repaired tissue is vulnerable, and its biomechanical features are less efficient than pre-injury (Leadbetter 1992).

As already described above, numerous secreted molecules complexly coordinate the

tendon healing process. First, intruding inflammatory cells produce certain inflammatory cytokines, like IL-6 and IL-1β. Then, numerous GFs secreted by cells at the injury site promote tissue repair. The following GFs support different phases of the healing process with specific molecular effects. FGF-2, bone morphogenetic proteins (BMPs)-12, BMP-13, and BMP-14, also known as growth and differentiation factors (GDF)-5, GDF-6, and GDF-7, respectively, TGFβ, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). When tendon cells are enabled during the healing process, they both synthesize and break down ECM components and thus take part in the steady and constant tendon remodeling process (Sharma and Maffulli 2005a, b; Voleti et al. 2012).

Favata et al. (2006) examined the regenerative properties of fetal sheep tendon and reported no adverse effect by transplantation into adult environment. However, they found that scar formation after surgery causes the most problems in adult tendon healing. In the inflammatory response, there was a remarkably great disparity between adults and fetal samples. The inflammatory infiltration in adult lesions was substantial, whereas such a reaction was obviously totally absent in 2- or 3-week fetal lesions. These findings support a related study about lesion healing in fetal skin (Favata et al. 2006). Moreover, samples of adult tendons showed higher levels of TGFβ-1, FGF-2, and CD44 at the lesion site, while those of fetal tendons had almost no alteration in injury response. The recruitment and proliferation of fibroblasts and macrophages, the upregulation of metalloproteinase inhibitors, the downregulation of proteinase activity, and angiogenesis are triggered by the cytokine TGFβ-1. Scar tissue formation after injury might partially result from higher amounts of TGFβ-1 at the lesion site.

In general, in contrast to fetal tendon regeneration, repaired adult tendons do not fully regain their former mechanical strength. The reduced integration of collagen fibers with a higher ratio of Collagen type III to Collagen type I results in limited strength of the repaired tissue in comparison to native tendon. Consequently, to compensate for lower unit mechanical strength, the tendon thickens and stiffens, thus decreasing tendon quality and functional activity as compared to a healthy tendon.

2 Application of Stem Cells, GFs, and Transcription Factors in Tendon Injury Therapies

2.1 Stem Cells

During the last decade, potential regenerative therapies based on cell and blood substrates attracted interest (Geburek and Stadler 2011; Goodship et al. 1994; Stewart and Stewart 2011).

In 2007, Bi et al. reported a new cell population in human tendons (from 8- to 12-year-old children with hamstring contractures) which were named tendon stem/progenitor cells (TSPCs) (Bi et al. 2007). According to this study, these TSPCs displayed the characteristics of classical adult MSCs: certain surface antigens, self-renewal, clonogenicity, and three-lineage adipogenic, osteogenic, and chondrogenic differentiation. Moreover, tendon-related genes such as SCX and TNMD were expressed by TSPCs, and they could generate tendon and enthesis-like tissues in vivo. TSPC populations were reported in human, equine, rabbit, rat, and mouse tendons (Haasters et al. 2011; Kohler et al. 2013; Lovati et al. 2012; Mienaltowski et al. 2013; Rui et al. 2010; Steinert et al. 2011; Tempfer et al. 2009; Zhang and Wang 2010).

It is still uncertain if TSPCs are a residual population of the embryonal tendon progenitors. Also further studies defining the exact function and location of TSPCs during tendon recovery are necessary, explaining their specific relation to tenoblasts and tenocytes. The clear distinction of TSPCs from tenoblasts and tenocytes is currently limited due to missing molecular markers. These markers would make an exact identification of TSPCs possible and thus their isolation of pure subsets of cell populations along the tendon differentiation cascade. Strained resources and

limited proliferation of these cells are clear disadvantages.

Several studies describing clinical effects after transplantation of autologous tenocytes in humans obtained from patella tendon biopsies have been published. In 2013 an autologous tenocyte injection for the treatment of severe, chronic resistant lateral epicondylitis was published as a pilot study (Wang et al. 2013). Later on, Wang et al. provided further evidence for the durability of a single autologous tenocyte injection into the common extensor tendon origin for the treatment of chronic resistant lateral epicondylitis (Wang et al. 2015). In this study tenocyte cell therapy significantly improved clinical function and magnetic resonance imaging (MRI) tendinopathy scores for up to 5 years. In another study, Bucher et al. performed a single injection of cultured tenocytes in patients with chronic recalcitrant gluteal tendinopathy also directly into the tendinopathic area (Bucher et al. 2017). These patients had also failed to respond to existing conservative treatments including physiotherapy. The authors came to the conclusion that cell injections for gluteal tendinopathy are safe, with improved and sustained clinical outcomes up to 24 months. In the same context, the Australian company Orthocell is providing tenocyte cell therapy for patients.

In addition to tenocytes, also MSC research studies address tenogenic differentiation, a field that possibly has potential in tendon regeneration (Deng et al. 2014; Jiang et al. 2016; Yin et al. 2013, 2016), and provide an alternative resource in cell therapies for tendon injury (Docheva et al. 2015; Gaspar et al. 2015). Furthermore, specific types of mature and differentiated somatic cells, like dermal fibroblasts, may be transdifferentiated into tenogenic phenotypes in addition to MSCs (Chen et al. 2016; Wang et al. 2016). All these approaches are dependent on efficient cell culture differentiation protocols for the generation of TSCs or tenocytes.

Recent advances in the immunobiology of MSCs have led to increased interest in their use as a new therapeutic modality to address chronic inflammation associated with fibrosis. MSCs have an immunosuppressive impact, which has been the reason for detailed examinations and reports, not only for its potential in organ transplantation (Mok et al. 2013; Singer and Caplan 2011; Usunier et al. 2014). The immunosuppressive effect of MSCs has been extensively described. The influence of MSCs hinders the G0/G1 phase of the cell cycle by blocking the T and B lymphocytes, which prohibits immunoglobulin production (IgA, IgG, and IgM) and B lymphocyte differentiation. In T lymphocytes, the polarity is varied from a pro-inflammatory T-helper Th1 cell state to an anti-inflammatory Th2 condition by the influence of MSCs (Corcione et al. 2006; Keating 2008). In dendritic cells, they function in maturation and differentiation and cause their tolerogenic state (Bifari et al. 2008).

Moreover, the cytotoxic activity of natural killer cells on HLA-1 negative cells (human leukocyte antigen-1) is hindered by MSCs, as they also limit the cytokine generation like tumor necrosis factor-α (TNFα), interferon-γ (IFN-γ), and IL-10 (Sotiropoulou et al. 2006). Thus, MSCs might have a great potential in therapies of inflammatory diseases.

The advantages of MSCs for immunomodulation in fibrosis therapy have been emphasized in many reports. Due to the minimized expression of the toll-like receptor triggered by MSCs, it is implied that they can reduce chronic inflammations (Linard et al. 2013). In many animal models, the infiltration by monocytes/macrophages, neutrophils, and lymphocytes was declined after MSC transplantation in the affected tissue (Choi et al. 2014; Lee et al. 2010; Linard et al. 2013; Ueno et al. 2013; Zhou et al. 2013a). These findings are supported by the diminished MCP-1 expression, as reported in certain cases (Zhou et al. 2013a). Furthermore, the molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which play a role in leukocyte-endothelial cell interactions, have been found under-expressed, which implies that they limit the inflammatory infiltration of cells (Zhou et al. 2013a). Due to the diminished expression of nitric oxide synthase posttransplantation of MSCs, it can be suggested that the activity of M1 macrophages is decreased (Linard et al. 2013). In a heart fibrosis model, a higher amount of anti-inflammatory M2

macrophages was found posttransplantation of MSCs (Ishikane et al. 2013). The fact that the IFN-γ expression was hindered by MSCs implies that IL-6 and TNF-*α* overexpression has a pro-inflammatory effect (Moodley et al. 2009). Certain studies report of a diminished TNF-*α* mRNA expression and protein concentration posttransplantation of MSCs and pro-fibrotic cytokines. In certain fibrosis models, an under-expression of IL-1*α*, IL-1β , and IL-6 can be found after the injection of MSCs (Asanuma et al. 2011; Bai et al. 2013; Lee et al. 2010; Linard et al. 2013; Moodley et al. 2009; Qiao et al. 2011; Semedo et al. 2009; Horton et al. 2013; Zhou et al. 2013a; Qi et al. 2014; Franquesa et al. 2012; Horton et al. 2013). Following MSC transplantation, the anti-inflammatory cytokines IL-4 and IL-10 show a higher expression, which implies a T-lymphocyte transition to the Th2 profile (Semedo et al. 2009). In a radiation-induced fibrosis model, a higher IL-10 concentration and expression were caused by MSCs in a similar way (Horton et al. 2013; Linard et al. 2013). After MSC transplantation, also less apoptotic events were reported in fibrotic tissues (Mohammadi Gorji et al. 2012; Semedo et al. 2009; Song et al. 2013; Zhang et al. 2011). Thus, MSC could foster and protect resident cells by enhancing their functionality and recovery. Saether et al. (2016) primed MSCs for 48 h using polyinosinic acid and polycytidylic acid increasing their secretion of IL-6 and PGE2 and administered 1×10^6 cells in a carrier solution at the time of injury. Applying these primed MSCs beneficially altered healing by affecting endothelialization, M2 macrophage presence, apoptosis, procollagen 1-α, and IL-1Ra levels (Saether et al. 2016). Thus altered key ligament healing events resulted in a more anti-inflammatory environment and improved healing.

It can be implied that treating tendon lesion with multipotent MSCs may directly affect tendon regeneration (Guest et al. 2010; Smith et al. 2013; Watts et al. 2011). Furthermore, it was shown that injected MSCs initiate a paracrine effect by supplying trophic mediators, GFs, and immunomodulatory, angiogenic, as well as anti-apoptotic substances or possibly integrate and synthesize tendon matrix and thus directly affect healing (Caplan 2009; Caplan and Dennis 2006; Herrero and Perez-Simon 2010; Lovati et al. 2012; Park et al. 2010; Raabe et al. 2013; Rehman et al. 2004; Richardson et al. 2007; Sorrell et al. 2009; Tan et al. 2012; Violini et al. 2009).

Which selective cell source, different kinds of MSCs or tenocytes, is the best for improved tendon healing remains unclear so far and might be dependent on the different stages of tendon healing (Alves et al. 2011).

When examining adipose-derived nucleated cell (ADNC) fractions and compared with adipose tissue-derived mesenchymal stem cells (AT-MSCs), it became obvious that ADNCs are a mixture of different cell types. Their benefit lies in direct and timely availability after harvest of tissue cells, without expenses for culturing. Also pericytes were proven to be multipotent and develop a subgroup of ADNCs (Koch et al. 2009). In contrast, culturing AT-MSCs produces a higher cell dose and thus hypothetically more effect (Peroni and Borjesson 2011). Which cell type has the greatest effect for tendon regeneration remains unclear to the authors (Koch et al. 2009; Nixon et al. 2008). In a collagenase model experiment with horses, the effect of ADNCs on tendon healing was diminished; however, it caused an improved tendon organization histologically and an augmented expression of cartilage oligomeric matrix protein (COMP) (Nixon et al. 2008).

AT-MSCs in contrast to bone marrow-derived mesenchymal stem cells (BM-MSC) are on the one hand multipotent and surpass in adipogenesis; on the other hand, they are not as effective in osteogenic and chondrogenic differentiation (Liu et al. 2007; Yoshimura et al. 2007). Adding IGF-1, TGFβ, or BMP-14 causes a tendon-related marker gene expression upregulation by AT-MSCs, including SCX and TNMD (Park et al. 2010; Schneider et al. 2011). When comparing AT-MSCs and BM-MSCs with tendon-derived cells, Kryger et al. (2007) detected similar scaffold adherence and proliferation potential and thus implied that AT-MSCs can be utilized as an alternative for regenerating tendon tissue (Kryger et al. 2007). In another animal

model, rabbit deep digital flexor tendons treated with AT-MSCs produced higher yield loads and energy absorption (Behfar et al. 2012). Summarizing, AT-MSCs are multipotent and commonly obtainable cells; they can easily be attained without morbidity risk and thus constitute an interesting origin for cells in tendon tissue engineering.

In equine orthopedics, bone marrow, adipose tissue, and umbilical cord blood are clinically the most relevant origins for MSCs (Alves et al. 2011; Taylor and Clegg 2011). Since adipose tissue is readily available to harvest, it is more beneficial than bone marrow. Besides, adipose tissue contains more MSCs, and its ability to proliferate AT-MSCs is increased (Burk et al. 2014); on top of that its cellular senescence is slower than that of bone marrow mesenchymal stem cells (BM-MSCs) (Alves et al. 2011; Kern et al. 2006; Vidal et al. 2007). As reported in a recent study, compared to BM-MSCs, umbilical cord blood and AT-MSCs express Collagen 1A2, Collagen 3A1, and DCN at the highest levels with the highest Collagen types 1A2 to 3A1 ratio (Burk et al. 2014), although a specific set of surface markers to characterize equine tenocytes was missing. The tendon markers COMP and SCX are highly expressed in AT-MSCs (Burk et al. 2014; Raabe et al. 2013). Moreover, several uncontrolled case series reported that AT-MSCs had already been administered to horses with tendinopathies and brought good results (Ricco et al. 2013). The implantation of AT-MSC into collagenase-induced superficial digital flexor tendon (SDFT) core lesions, for example, brought good results in a controlled in vivo experimental study: improved tendon fiber organization, decreased inflammatory infiltrate, and higher Collagen type I gene expression as compared to the control limbs. However, in clinical parameters and with B-mode ultrasonography, no differences were monitored (Carvalho et al. 2014). A limited progression of SDFT lesions and better organized Collagen fibrils, less inflammation, and increased vascularity were the results of a further experiment from the same group using the collagenase-gel model of tendinopathy with intralesional AT-MSC treatment suspended in platelet concentrate (Carvalho et al. 2013).

In a current study (Geburek et al. 2017), it was demonstrated that one single administration AT-MSCs suspended in inactivated autologous serum improved Collagen cross-linking. The content of hydroxylysylpyridinoline (HP) in the AT-MSC-serum treatment group was closer to the normal situation. This observation might be beneficial for improved long-term effects of AT-MSC treatment in tendon injury. Both the expression of Collagen types I and III during tendon repair can be affected by MSCs and autologous serum (Burk et al. 2014; Geburek et al. 2015; Majewski et al. 2009). Due to the fact that the window of optimum expression of Collagen types I to III ratio during the remodeling phase was not precisely defined (Patterson-Kane and Firth 2009), it is still uncertain, if the differentiation of Collagen types was helpful to distinguish which kind of treatment was more beneficial in the current study.

Xu et al. (2016) analyzed secretory proteins that were extracted from conditioned media of AT-MSCs by proteomics. In the matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF)/TOF spectrometer analysis, the following proteins were identified (Xu et al. 2016): VEGF; macrophage colony-stimulating factor; stromal cell-derived factor-1; TGF-β; TNF-α; IL-1, IL-6, and IL-8; hepatocyte growth factor; IGF-1; MMP-2; FGF-1; granulocyte macrophage colony-stimulating factor; pigment epithelium-derived factor; metalloproteinase inhibitor 1; plasminogen activator inhibitor (PAI); CTGF; Collagen-1; Collagen-6; and fibronectin (FN). The UniProt database gave the following molecular function information: 52.4% of all proteins were GFs, while 38.1% were cytokines and 19% mitogens. The biological process analyzation made obvious that 19% of all protein types functioned in angiogenesis, 14.3% in inflammatory response, 14.3% in cell adhesion, 9.5% in cell differentiation, 9.5% in chemotaxis function, and 9.5% in acute-phase response (Xu et al. 2016).

The first clinical trial for the application of autologous MSCs in Achilles tendinopathy has been listed by the US National Institutes of Health (identifier: NCT02064062).

The induction of a pluripotency status in somatic cells by direct reprogramming could be one further option to produce large amounts of TSC and tenocytes in the future. Somatic cell reprogramming has been employed with different kinetics and health effects utilizing various somatic sources (Okita et al. 2011). It is feasible in the future that patient-specific induced pluripotent stem cells (iPSCs) could supply for large amounts of transplantable tendon progenitor cells, which could be used to analyze tendon development and mechanisms of adult tenogenesis, also comparing repair and regeneration. Mesodermal precursor and tendon cells from various stages of tissue repair and tendon regeneration could originate from iPSCs. In this context still critical is the generation of iPSCs and the development of stable differentiation protocols which is an important milestone in stem cell biology. Up to now, iPSCs are difficult to control and display a genetic and epigenetic instability.

2.1.1 Other Important Cell Sources for Tendon Healing

Loiselle et al. detected an enormous proliferation of fibroblast-like cells in the wound healing stage of tissue granulation. Most of these proliferating cells originated from resident tenocytes (Loiselle et al. 2016). Hasslund et al. observed that autografts have a direct influence on the reduction in granulation at the lesion site (Hasslund et al. 2008). In a study about flexor tendon healing, Cadby et al. started to clarify distinct functions of peritenon and tendon core cells (Cadby et al. 2014). During healing, the proliferation capacity of cells originating from the peritenon was greater, but their probability to transform into myofibroblasts was higher than for local tendon cells. Moreover, a flexor tendon basement membrane, which is necessary for tendon gliding, was detected (Taylor et al. 2011b). This membrane consisted of Collagen type IV and laminin over a keratinized epithelium. A mutation of the Collagen type IV alpha 1 (COL4A1) protein caused a discontinuity of the basement membrane organization and integrity and induced the spontaneous formation of adhesions between tendon and surrounding tissue. Lately experiments had promising outcomes, when TGFβ was directly inhibited by certain small molecules that were applied directly or by modulating the downstream signaling. A nanoparticle-encapsulated TGFβ micro RNA was utilized by Zhou et al. for TGFβ suppression (Zhou et al. 2013b). In a chicken model, this suppression caused reduced adhesion with minor strength of the regenerated tendon. This outcome was supported by an experiment applying the antisense oligonucleotide (ASO) for TGFβ suppression, with certain parts of TGFβ signaling and Smad3. The gliding function was significantly improved when TGFβ and CTGF were inhibited in relation to control ASOs, but the strength could not be improved. Intriguingly, the gliding function and strength could be enhanced by administering a Smad3 ASO (Loiselle et al. 2015). As a delivery model, Basile et al. utilized a devitalized and acellular allograft tendon that was infected with a recombinant adeno-associated virus, which expressed BMP-14 (Basile et al. 2008). In relation to the adeno-associated virus control, the graft could be repopulated and scar tissue could be minimized, while the gliding features improved.

Furthermore, substantially enhanced gliding function in canine flexor digitorum profundus tendons could be accomplished by Zhao et al. (2014) when combining lubricin with hyaluronic acid and MSCs that were BMP-14 stimulated (Zhao et al. 2014 and Loiselle et al. 2016).

These outcomes show that gene therapy approaches in tendon regeneration with allografts or tissue-engineered tendons are practicable.

2.2 GFs in Tendon Development and Healing

During the different healing phases after tendon lesion, the production of numerous GFs is triggered (Evans 1999; Yang et al. 2013), which causes augmented cellularity and tissue volume (Sharma and Maffulli 2006). Especially, during the initial healing stages, GFs are increasingly

expressed (Heisterbach et al. 2012; Wurgler-Hauri et al. 2007). The GFs FGF-2, BMP-12, BMP-13, BMP-14, CTGF, IGF-1, PDGF, TGFβ, and VEGF all play distinct key roles in tendon healing (Chen et al. 2008; Kobayashi et al. 2006; Molloy et al. 2003; Wurgler-Hauri et al. 2007).

Below, these factors are briefly described, and in vitro and in vivo studies that examined the function of GFs involved in tendon healing are reported in more detail.

FGFs Intratendinous healing in the patellar tendons was increased by FGF-2 (Chan et al. 1997). Brent and Tabin could demonstrate that FGF acts directly on the somatic tendon progenitors through the E26 transformation-specific (ETS) transcription factor PEA-3 and ERM to regulate SCX expression (Brent and Tabin 2004). Recently, Havis et al. demonstrated that during chick limb development, TGFβ and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation (Havis et al. 2016). The activities of FGF/ERK mitogen-activated protein kinase (MAPK) and TGFβ/Smad2/Smad3 signaling pathways are decreased in tendons under immobilization conditions. Application of FGF-4 or TGFβ-2 ligands prevented SCX downregulation in immobilized limbs. It has been observed that TGFβ-2 but not FGF-4 prevents TNMD and thrombospondin-2 (THBS-2) downregulation under immobilization conditions. Chang et al. reported upregulated FGF-2 mRNA in mature tenocytes, fibroblasts and in inflammatory cells that surrounded the healing site in the tendon sheath (Chang et al. 1998). Since the FGF-2 level is increased during the initial healing process, it can well support the early events in tendon healing (Muller et al. 2015) but might also reinforce scarring by strongly stimulating the proliferation of fibroblasts from the endotenon, epitenon, paratenon, and tendon sheath.

BMPs BMP signaling generally inhibits tendon differentiation during development while inducing cartilage. Selected members of the BMP family (BMPs-12, -13, and -14) drive tendon differentiation (Chhabra et al. 2003; Clark et al. 2001; Mikic et al. 2008, 2009). Especially, specific members of the BMP family were reported to promote tenocytic differentiation from multipotent MSCs (Lee et al. 2011; Wolfman et al. 1997; Yu et al. 2007). The GFs BMP-12, BMP-13, and BMP-14 induce mitogenesis and are proven tenogenic factors for MSCs in vitro and in vivo (Park et al. 2010; Wolfman et al. 1997). At the beginning of tendon regeneration, BMPs are enhanced and gradually diminish in the later stages of healing (Heisterbach et al. 2012; Wurgler-Hauri et al. 2007). BMP-2 functions in enthesis, the anatomical transition from tendons to bones. Within a tendon with qualities similar to the enthesis, BMP-2 can induce new bone formations. Nevertheless, this kind of bone formation in intratendinous healing is barely adverse (Chen et al. 2011; Hashimoto et al. 2007; Rodeo et al. 1999).

Out of over 20 BMP members, BMP-12 seems to be the tendon inducer with the highest potential so far (Wagner et al. 2010). The transfer of the BMP-12 gene enhances the regeneration of lacerated tendons (Lee et al. 2011; Lou et al. 2001) and the recovery of Achilles tendons (Majewski et al. 2008). MSCs that were transfected or stimulated with BMP-12 in vitro were observed to generate a sufficient amount of tenocyte-like cells (Violini et al. 2009).

Shen et al. examined the influence of BMP-12 and BMP-14 on adipose stromal cell (ASC) differentiation in vitro (Shen et al. 2013). The expression of the tendon markers SCX and TNMD, at both mRNA and protein levels, was efficiently augmented by BMP-12 in canine ASCs. These findings agree with the fact that in mouse ASCs, SCX promoter-driven GFP and TNMD protein expression was induced by BMP-12. However, in ASCs, the expression of the cartilage matrix gene aggrecan was increased by BMP-12, whereas the resultant amounts stayed significantly below those of tendon fibroblasts. Furthermore, the bone marker expression osteocalcin was decreased by BMP-12, while the osteogenic runt-related transcription factor 2 (RUNX-2) was unreduced. In

comparison with BMP-12, the impact of BMP-14 appeared resemblant, with slightly less potency and selectivity. It is a fact that BMPs signal through the pathways of Smad (canonical) and MAPK (noncanonical). These outcomes suggest that tenogenic ASC differentiation is induced by BMP-12 through the Smad1/Smad5/Smad8 pathway.

Otabe et al. aimed on testing the effects of tendon-specific transcription factors on BM-MSCs (Otabe et al. 2015). BM-MSCs were treated with BMP-12 and lentiviral Mohawk (MKX), SCX, and epidermal growth factor (EGF)-1 gene vectors to determine to what extent the expression profiling of mesenchymal tenogenic lineage differentiation and ECM-related genes was affected. Contrasting the overexpression of the MKX and SCX gene of BM-MSCs, their TNMD gene expression was similar for both factors; however, the COL1A1 mRNA and protein expression of MKX was stronger. The findings of Otabe et al. imply that the tenogenic differentiation of BM-MSCs is triggered by MKX and SCX in combination with BMP-12 and thus indicate a future potential of this approach for new regenerative therapies. According to a further study, chemically modified mRNA caused tendon regeneration (Groth et al. 2017). The administration of a BMP-7-encoding chemically modified mRNA triggered a substantially enhanced BMP-7 protein expression and minimized the Collagen type III production.

EGF EGF is a potent mitogen that participates in MSCs and fibroblast proliferation and is also involved in the initial phase of tendon healing. Besides MSC proliferation, EGF treatment also preserves early progenitors within a MSC population and increases the paracrine activity of stem cells (Hebert et al. 2009; Woo et al. 2007). Tsubone et al. observed that EGF, IGF, and FGF-2 were not localized in tenocytes but were present in inflammatory cells surrounding the repair site in canine flexor tendon after laceration in vivo (Tsubone et al. 2004).

CTGF Unlike the factors described above, during the healing process of chicken flexor tendons, CTGF displays a continuing accumulation in gene expression for more than 21 days (Chen et al. 2008). In a rat model, Wurgler-Hauri et al. examined a supraspinatus injury and reported a moderate CTGF expression in the insertion and the mid-substance area during all instants of time (Wurgler-Hauri et al. 2007). Liu et al. (2015) could show that CTGF positively regulates BMP-12 inducing tenogenic differentiation of tendon stem cells and signaling (Liu et al. 2015). Overexpression of CTGF increased the expression of SCX and TNMD as well as tendon proteins Collagen type I and tenascin-C (TN-C) in TSCs compared to non-treated control cells with or without simultaneous BMP-12 stimulation, while knockdown of CTGF expression decreased the expression of SCX, TNMD, Collagen I, and TN-C. Chemical cross-linking experiments demonstrated a direct interaction between CTGF and BMP-12. The authors concluded that BMP-12 plays a crucial role in tenogenesis via the Smad1/Smad5/Smad8 pathway and CTGF.

Lee et al. identified a rare fraction of CD146-positive tendon cells that exhibited clonogenic capacity, as well as multilineage differentiation ability (Lee et al. 2015). These tendon-resident CD146+ stem/progenitor cells were selectively enriched by CTGF delivery in the early phase of tendon healing, followed by tenogenic differentiation in the later phase. The time-controlled proliferation and differentiation of CD146+ stem/progenitor cells by CTGF delivery successfully led to tendon regeneration with densely aligned collagen fibers, normal level of cellularity, and functional restoration.

Tarafder et al. (2017) could demonstrate that the delivery of CTGF into full-transected rat patellar tendons significantly increased the number of CD146+ TSCs, leading to enhanced healing. In parallel, CTGF delivery showed anti-inflammatory roles of CTGF-stimulated TSCs that are likely associated with improved tendon healing (Tarafder et al. 2017).

IGF-I In vitro, the matrix synthesis and cell proliferation of tenocytes was as well induced by IGF-1 (Abrahamsson 1991; Abrahamsson et al. 1991). A higher expression of IGF-1 mRNA and proteins was detected in healing ligaments of rabbits 3 weeks post-injury and in tendons of horses 4–8 weeks post-injury (Dahlgren et al. 2005; Sciore et al. 1998). Over the formation and remodeling healing phase, IGF-1 appears to be especially important. The synthesis of the ECM with Collagen is enhanced, and tenocyte migration is increased by IGF-1 (Trippel et al. 1993).

Provenzano et al. analyzed if systemic administration of IGF-I improves healing in collagenous extracellular matrices (Provenzano et al. 2007). The growth hormone (GH) alone did not result in any significant improvement contrary to their hypothesis, while GH in combination with IGF-I produced remarkable improvement in hindlimb tendon healing.

Farnebo et al. hypothesized that combinations of FGF-2, IGF-1, and PDGF homodimer BB would improve cellular proliferation and survival of ASCs seeded to a tendon ECM gel (Farnebo et al. 2017). Furthermore reseeding with ASCs stimulated endogenous repopulation of the gel in vivo and may be used to further augment tendon healing.

PDGF In healing tendons, higher levels of PDGF were detected (Duffy Jr et al. 1995). Furthermore, PDGF triggered proteoglycan, Collagen, non-collagenous protein, and DNA synthesis (Yoshikawa and Abrahamsson 2001). Collagen cross-linking and hyaluronic acid were enhanced by PDGF treatment and resulted in improved functional movement; however, the tensile properties of the regenerated tendon were not improved (Thomopoulos et al. 2009). Increased PDGF-receptor-β expression was reported by Chan et al., continuing more than 6 months after tendon lesion, which suggests a possible vital role of PDGF for the complete tendon regeneration phase (Chan et al. 2006). Suwalski et al. observed accelerated Achilles tendon healing by PDGF gene delivery with mesoporous silica nanoparticles (Suwalski et al. 2010). GFs produce not only positive results in intratendinous lesions; they can also like PDGF increase the breaking load of the femorotibial ligament (Batten et al. 1996).

TGFβ The signaling pathways underlying regenerative and fibrotic tendon healing have yet to be elucidated, but one attractive candidate is the TGFβ/BMP family. During development, TGFβ signaling is essential for tendon formation, and it is well established that TGFβ can induce tendon markers in cell culture. Interestingly, TGFβ can also drive chondrogenic differentiation, and injection of TGFβ ligands has been used to induce cartilage deposits in tendon, suggesting a potential role for TGFβ in ectopic cartilage formation after tendon injury as well (Bell et al. 2013; Johnstone et al. 1998; Pittenger et al. 1999).

TGFβ in particular stimulates ECM production and enhances the production of Collagen types I and III by all three isoforms, TGFβ-1, TGFβ-2, and TGFβ-3; furthermore it functions in cell migration and mitogenesis (Klein et al. 2002). TGFβ activity and increased expression levels are detected during the tendon healing process (Chang et al. 2000; Natsu-ume et al. 1997). A higher expression of TGFβ-1 mRNA was also found in resident tenocytes and infiltrating cells from the encircling tendon sheath (Chang et al. 1997). Accordingly, at the repair point, an upregulated TGFβ-1/TGFβ-3 receptor (CD 105; endoglin) expression was also reported (Ngo et al. 2001). A biphasic TGFβ expression pattern was detected by Juneja et al., who confirmed the highest points of TGFβ-1 expression early in the process and of TGFβ-3 expression late in the process of healing (Juneja et al. 2013). Heisterbach et al. also found early and late heights of TGFβ-1 expression (Heisterbach et al. 2012). Other findings suggest that TGFβ-1 causes a stronger formation of fibrotic scars and adhesions (Katzel et al. 2011). In a rabbit model, Chang et al. found that adhesions were reduced when applying anti-TGFβ-1 antibodies, but not by adding antibodies against the isoform TGFβ-2 (Chang et al. 2000). Therefore, in the context of injury, TGFβ-1 has thus to be implicated as a potent inducer of fibrosis.

It could therefore be important for a successful application of TGFβ in tendon healing to define the appropriate timing, isoform doses, and combinations or even antibodies to block TGFß-1 function for fibroblast proliferation.

VEGF Angiogenesis plays a vital role in tendon degeneration in cases of impaired blood supply, as well as during repair; thus an optimal capillary permeability is preferable (Fenwick et al. 2002). The angiogenesis in tendon healing is caused by VEGF (Petersen et al. 2003), and VEGF activity increases in the proliferative and remodeling phase of wound healing in particular. The VEGF mRNA level was the highest 10 days after surgery in a canine model of tendon transection carried out by Zhang et al. (2003). Kaux et al. observed that a local injection of bioactive recombinant VEGF-111 improved the early phases of the healing process of rat tendons after a surgical section (Kaux et al. 2014). Mao et al. modulated digital flexor tendon healing by VEGF gene transfection in a chicken model (Mao et al. 2017). Here, delivery of AAV2-VEGF significantly increased the Collagen type III and MMP2 expression and ultimate strength of the healing tendons postoperatively.

2.2.1 Effects of Different GFs on Tendon Healing

Numerous studies were published with the goal to understand the impact of GFs on tendon biology in vitro and on tendon healing in vivo. In in vivo studies, GFs were administered by local injection, percutaneously or operatively implanted in scaffolds or even in the form of suture material that contains GFs (Hamada et al. 2006; Rickert et al. 2001; Uggen et al. 2010). After local injection, the GFs quickly resolve; however, they remain at the site of injection for longer if scaffolds or coated suture materials are used.

Only few studies report about GF release by coated suture materials and scaffolds in tendons, but several trials examined the local GF application. Anaguchi et al. reported that the local injection of TGFβ into the healing site of patellar tendons in rats strongly increased the load to failure (Anaguchi et al. 2005). Flexor tendons of rabbits treated with VEGF showed similar results. Early in the healing process, the TGFβ expression was highly increased in this study. It could not be found, whether this effect was accomplished by the VEGF therapy itself or by the VEGF-triggered increased TGFβ expression or by both (Chen et al. 2004).

In canine flexor tenocytes, the deposition of fibronectin was enhanced during the combination of FGF-2 and PDGF treatment, with enlarged parts of the provisional matrix and angiogenesis/revascularization (Harwood et al. 1999). It is interesting that native cells from different areas of the tendon tend to show various reactions to TGFβ administration. In comparison to epitenon or tendon sheath cells, in endotenon cells, the collagen type I expression is downregulated and the collagen type III expression upregulated (Klass et al. 2009). It is feasible that, when cells in the endotenon upregulate collagen type III and downregulate collagen type I, this starts TGFβ-induced tendon healing (Zhang et al. 2004). PDGF, FGF-2, or in combination induced an enhanced cell proliferation and collagen production as well. In a study by Thomopoulos et al., the same positive effect could not be found for VEGF and BMP-2 (Thomopoulos et al. 2005).

Goncalves et al. performed a study to better understand the role of GFs in modulation of stem cell tenogenesis. Specific GFs that play a key role in tendon development and regeneration, including EGF, FGF-2, PDGF-BB, and TGFβ-1, were used to examine the tenogenic differentiation potential of human amniotic fluid stem cells and human adipose-derived stem cells (hASC). The tendon-related genes Collagen type I and III, DCN, TN-C, and SCX and several proteins of the tendon ECM like Collagens I and III and TNC were screened to analyze the reaction of stem cells to biochemical stimuli. It was detected that EGF and PDGF-BB induced in hASC a tenogenic expression profile, although GFs apparently had no effect on tendon ECM protein synthesis (Goncalves et al. 2013). The examined GFs were obviously not substantial for biochemical in vitro tenogenesis, although the genetic expression levels of tendon-associated markers were

increased, and TN-C and Collagen I were also examined in basic medium.

These findings imply that multiple GFs and their administration at specific time points are essential for the complicated elaborated tenogenic differentiation pathways, as well as combined multifactorial stimuli that originate from the specific natural surroundings of the tendon. Probably, mechanical stimulation would have also an effect on the studied stem cells in addition to the biochemical signals within the culture medium.

According to the proteomic analysis by Yu et al. (2016), most of the GFs in tendon development and healing are secreted by AT-MSCs. GFs which are not expressed by AT-MSCs include BMPs and EGF, which might be relevant candidates for additional pharmaceutical substitution in relevant phases of tendon healing in addition to MSC cell therapy.

Platelet-Rich Plasma (PRP) PRP consists of autologous blood containing a platelet concentration that is 3–4 times higher than at baseline. These platelets include alpha granules with numerous GFs involved in cell recruitment, proliferation, and angiogenesis and thus are convincing for medical indications (Galatz et al. 2015).

Quite a few varying products can be obtained commercially. The evaluations in certain studies demonstrated the function of PRP in tendinopathies and tendon repair. PRP possibly plays a distinctive role in the future treatment of tendinopathies, like lateral epicondylitis and Achilles tendinitis, although the beneficial effect of PRP in rotator cuff repairs has not been proven yet (Castricini et al. 2011; Randelli et al. 2011; Theodoropoulos 2011). Out of three randomized trials, only one reported a minor improvement in tendon integrity with no clinical distinction as against untreated repairs; two reported no benefit (Gumina et al. 2012; Taylor et al. 2011a; Weber et al. 2013). The perfect timing for supporting the GFs might be a critical factor in the practical viability of PRP. The alpha granules release these factors within 1 hour upon activation of PRP. The half-life of most GFs lasts just a few minutes (Foster et al. 2009). From day 7 to 14 after tendon injury, the majority of tendon healing studies report a progression after the administration of PRP (Galatz et al. 2006).

It is rather crucial for accomplishment to support the GFs at the specific peak in metabolic activity. Since the platelets are in an absorbable fibrin matrix, which causes a slower and more controlled release of factors as the fibrin matrix can absorb, a constant delivery utilizing platelet-rich fibrin matrix is imperative (Barber et al. 2012; Bergeson et al. 2012).

In spite of that, advanced studies for optimizing the PRP application are indispensable; nevertheless, it has not been proven yet that providing GFs alone massively improves scarless healing and regeneration of tendons.

2.2.2 ECM and Tendon Regeneration

For cellular components, the ECM supports substantial physical scaffolding, and furthermore, it induces essential biochemical and mechanical stimuli necessary for the morphogenesis of tissues (Badylak 2007; Claudio-Rizo et al. 2017; Theocharis et al. 2016). The ECM composition of the human tendon has been recently identified (Sato et al. 2016).

Artificial ECM patches are utilized to repair the tendon thus reinforcing the strength of the recovery. The patch could advance new growth and differentiation as a scaffold, thus possibly supplying cells and GFs in the future. Dermis and intestinal submucosa together with synthetic substances are tissues currently available.

Kaux et al. (2015) reviewed preclinical studies that showed promising results: the administration of hyaluronic acid (HA) minimized adhesions and inflammations, enabled tendon gliding, and improved the tendon organization architecture (Kaux et al. 2015). Certain promising short-term clinical results about pain analysis and function seem to support these laboratory findings. Nevertheless, according to Kaux et al., further controlled randomized studies are required (Kaux et al. 2014).

By contrast various commercial HA gels applicable for human fetal progenitor tenocyte resuspension in the Ostenil Tendon were examined by Grognuz et al. in a study in 2016. They found that the HA gel was a reasonable compromise to provide a formula for good cell survival and adequate rheological features for sustaining a cell delivery system (Grognuz et al. 2016).

In healthy tendons, an acute inflammation was caused by the injection of HA into the tendon, which had adverse pathological effects, as reported by Wu et al. in 2017. Even 42 days after the injection, the HA-induced acute tendon injury did not only result in histopathological changes, and clusters of ED1+ and ED2+ macrophages, however also in IL-1β expression and neovascularization. Thus, Wu et al. suggested to avoid of the administration of HA injections into injured tendons (Wu et al. 2017).

An explorative trial investigated the application of acellular human dermal matrix, which was promising for improved results and more successful healing (Barber et al. 2012). More trials with dermal products obtained good results in histologic findings on the second look; however, they lacked a control group (Gupta et al. 2012). Altogether and regardless of initial excitement for this new method of treatment, a regular application cannot be recommended due to lack of sufficient evidence. Even in the animal model, there was no substantial proof for tendon regeneration or scarless healing reported with these products (Gupta et al. 2012).

In a new model using micro-tissue, Foolen et al. (2017) looked at scarring in the tendon healing process and found that tissue alignment improved the capacity of tendon-derived cells to remodel. Cells located in aligned tendon tissue without scars demonstrated an outstanding ability to remodel tissue by their higher gene expression levels of collagen, MMPs and tissue inhibitors of MMP. According to Foolen et al., they also had more pronounced connexin43 gap junctions than the isotropic tissue with scars. These findings give insight into the limited ability of tenocytes to heal chronic tendinopathies, when ECM is deranged due to pathologic processes (Foolen et al. 2017).

Future studies will therefore elucidate the specific activities of GFs and their downstream signaling and interactions in combination with cell transplants and aligned matrices in tendon regeneration, fibrosis, and heterotopic ossification.

2.3 Transcription Factors

It has been reported that many transcriptional factors such as Six1 and Six2, EphA4, Eya1 and Eya2, follistatin, TN-C, MKX, and SCX regulate the development and formation of tendons during embryogenesis (Aslan et al. 2008; Jelinsky et al. 2010; Onizuka et al. 2014).

Latest studies assign an important role to the transcription factors homeobox SCX (Schweitzer et al. 2001), MKX (Ito et al. 2010); (Liu et al. 2010), early growth response proteins 1 and 2 (EGR-1 and EGR-2) (Derby et al. 2012; Guerquin et al. 2013), and TNMD during tenogenesis (Oshima et al. 2003).

SCX The class II basic helix-loop-helix transcription factor SCX has attracted special notice in tendon development and regeneration (Liu et al. 2014). TSC and differentiated tendon cells express SCX. In the developing limb buds and the syndetome, a somatic compartment at the dorsolateral edge of the sclerotome, SCX is observed at E10.5 in mice (Brent et al. 2003; Pryce et al. 2007; Schweitzer et al. 2001). During later phases of tendon formation, SCX expression is constantly found (Schweitzer et al. 2001). Severe tendon disruptions are caused by SCX gene knock in mice (Murchison et al. 2007). A failure in tendon stem cell condensation and a tendon differentiation defect and even the loss of tendons transmitting musculoskeletal force in limbs, tail, and trunk can be caused by conditional knockout of the SCX gene in mice (Murchison et al. 2007).

The primary role of the transcription factor SCX is determining the fate of MSCs toward tenocyte differentiation (Li et al. 2015). A murine embryonic fibroblast cell line (C3H10T1/2) was utilized as a model system to prove the substantial expression of SCX during the early phase of BMP-12-triggered tenocytic differentiation. Following induction, SCX

directly transactivates genes related to the tendon lineage like TNMD and hereby suppresses osteogenic, chondrogenic, and adipogenic capabilities. It thereby triggers C3H10T1/2 cells to differentiate into the specific tenocyte-like lineage, thus removing plasticity for other lineages. It is also reported that the mechanical loading-mediated tenocyte differentiation follows a similar pathway. BMP-12 and the cyclic uniaxial strain act in a familiar way here, activating the signal transducer Smad8 and thus enlarging the maximal response. These findings give specific understanding how far both chemical and physical signals induce the determination of multipotent stem cells to the tenocyte lineage.

The TNMD induction by SCX in BM-MSCs has already been demonstrated (Alberton et al. 2012). SCX gene transfer increased the impact of BM-MSC transplantation in a rotator cuff injury model, also enhancing its mechanical properties (Shukunami et al. 2006).

The outcomes of these experiments emphasize the key role of SCX in the development of tendons and imply its critical role in determining cell fate of adult MSCs and their ability to differentiate into tenocytes.

MKX MKX, an atypical homeobox protein, is counted to the Iroquois (IRX) family-related class. As a "three-amino acid loop extension" (TALE), MKX has the untypical characteristics of those homeobox proteins: in the loop region between helix I and helix II of the homeodomain, there are three extra amino acids (Anderson et al. 2006). Anderson et al. (2006) detected MKX transcription in discrete premyogenic cell populations of the somite when employing in situ hybridization. They also proved MKX transcription in mesodermal structures including the condensing prechondrogenic mesenchymal cells of the axial skeleton and the pretendinous cells of the tail and limbs.

In tendons and ligaments, the MKX transcription factor plays a key role in tenogenic differentiation of BM-MSCs and homeostasis. Viable, fertile MKX (-/-) mice with regular body weight were generated (Ito et al. 2010). Abnormalities appeared in size of fiber bundles and weaker tensiles, and they had smaller, hypoplastic tendons. Contrasting MKX(-/-) against wild-type embryos in electron microscopic analysis revealed a constantly inferior Collagen fibril diameter. The soluble Collagens in Achilles and tail tendons of MKX(-/-) mice were smaller. According to Ito et al., Achilles and tail tendons were found with less type I Collagens 1A1 and 1A2 (Ito et al. 2010). The Collagen assembly factor DCN was found downregulated, and the positive regulator of Collagen type I SCX was higher expressed. Ito et al. thus suggested an essential role of MKX in tendon maturation (Ito et al. 2010).

As reported in a recent study, SCX and MKX bind to Smad3 which transmits TGFβ signaling (Berthet et al. 2013). After tendon injury in Smad3-deficient mice, limited mechanical strength and irregular Collagen deposition were observed (Katzel et al. 2011).

Suzuki et al. utilized clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to engineer Mohawk mutant rats. These rats developed systemic hypoplasia of tendons comparable to MKX(-/-) mice and showed an earlier heterotopic ossification of the Achilles tendon. When examining the tendon-derived cells (TDCs), it became obvious that the lack of MKX advanced the process of chondrogenic and osteogenic differentiation. The MKX overexpression, however, caused suppression of the chondrogenic, osteogenic, and adipogenic differentiation. Moreover, the chondrogenic differentiation was triggered by the mechanical stretch stimulation of MKX(-/-) TDCs, which also caused tenocyte formation (Suzuki et al. 2016).

In the ChIP-seq of MKX overexpressing TDCs, substantial peaks were shown in chondrogenic differentiation-related genes, such as SRY-box gene (SOX)-5, SOX-6, and SOX-9, and in tenogenic-related genes like COL1A1 and COL3A1. These findings prove the dual function of MKX, comprising enhancing tendon differentiation and inhibiting chondrogenic/osteogenic differentiation. A practicable basis for tendon physiology and tissue engineering is supplied by the molecular MKX network.

Due to these findings and those of Otabe et al. (2015), it can be understood that MKX might be

one of the factors capable of inducing TNMD expression independent of SCX, at least in human BM-MSCs, while SCX is the dominant inducer of TNMD (Otabe et al. 2015). It thus appears that MKX and SCX function independently, despite possibly having similar effects on BM-MSCs at a later tenogenic differentiation phase.

The co-transfection of adenoviral constructs of MKX and SCX was also examined by Otabe et al. However, no favorable effect on tenogenic gene expression was found. The sequential co-transfection of MKX and SCX at perfect timing should be further examined in the future, since MKX is considered to function at a later tenogenesis stage than SCX (Ito et al. 2010; Liu et al. 2014).

Consequently, MKX in addition to SCX is regarded as a key regulator in tenogenic differentiation.

2.3.1 New Tendon Regeneration Model with Specific Cell Mechanisms

Since the molecular mechanisms underlying tendon regeneration are not well comprehended up to now, Howell et al. found a new model utilizing neonatal mice (Howell et al. 2017). Howell et al. demonstrated in his tendon regeneration model the capability of neonate mice tendons to form a completely new Achilles tendon after injury. This "neo-tendon" differentiated along the tendon lineage and fully recovered gait and mechanical functions and properties. This neonate tendon can thus regenerate and fully restore former tendon post-injury, as against adult tendons, which heal with a fibrovascular scar, aberrant differentiation toward cartilage and bone, and prolonged functional impairments.

The inner recruitment of SCX-lineage cells that play a key role when tracing the lineage of neonatal cellular healing mechanism, but lack in adults, was observed. In contrast, the adult SCX-lineage tenocytes are not recruited into the lesion area but are transdifferentiated into ectopic cartilage. While tenogenic cells are missing, permanent scar formation triggered by extrinsic αSMA-expressing cells continues. Altogether, these findings proved an interesting implementation for tendon healing and revealed new cellular mechanisms of regenerative and non-regenerative tendon healing. At any time post-injury, tendon markers were not significantly upregulated in adult tendons, as the analysis showed. Furthermore, at day 14, SCX and MKX were downregulated.

In summary, these outcomes demonstrate how the transient expression of fibrotic markers and succeeding tendon-specific differentiation affect the neonatal tendon regeneration progress. However, during neonatal healing stages, an aberrant differentiation into alternative mesenchymal lineages could not be observed.

EGR-1 EGR-1 was recently identified as a transcription factor involved in tenogenesis (Guerquin et al. 2013). These authors detected that tendon differentiation is triggered by the transcription factor EGR-1, which also supports tendon healing. In adult EGR-1–/– mice, the tendons were found deficient in tendon gene expression including SCX, COL1A1, and Col1A2, and their mechanical strength was limited. In postnatal tendons, the collagen type I production is also triggered by EGR-1 via direct regulation. In postnatal mouse tendons, EGR-1 was recruited to COL1A1 and COL2A1 promoters in vivo. In a mouse tendon lesion model, EGR-1 was necessary for regular gene response after injury of the Achilles tendon. The MSCs were programmed to tendon lineage after forced EGR-1 expression, which also supported the generation of in vitro MSC-engineered tendons. After Achilles tendon injury in a rat model, the generation of tendon-like tissue was enhanced by the administration of EGR-1-producing MSCs. It was proven that the capability of EGR-1 to promote tendon differentiation in part depends on TGFβ-2 mediation. As reported in this study, EGR-1 plays a role in the generation and regeneration of adult tendons and thus is a probable target for future tendon regeneration methods (Guerquin et al. 2013).

The expression levels of EGR-1 and EGR-2 did not change after an adenoviral overexpression

of SCX and MKX in BM-MSCs (Otabe et al., data not shown), which suggests that EGRs are not downstream of SCX and MKX.

TNMD The transmembrane surface molecule type II TNMD also referred to as tendin, or myodulin, is highly expressed in tendons and serves as a marker gene for tendinous and ligamentous lineages. TNMD expression is most commonly found in avascular tissues and specifically in dense conjunctive tissue like tendons or ligaments, heart valve, eye, and fat tissue (Oshima et al. 2003; Saiki et al. 2009; Shukunami et al. 2006). TNMD shares high similarity with chondromodulin-1 (Chm1) and is important for tendon maturation with key implications for the residing TSPCs.

The TNMD protein consists of a highly conserved C-terminal domain with 8 cysteine residues, which include a BRICHOS domain consisting of a conserved sequence of about 100 amino acids and a supposed protease recognition site RXXR. Here, the furin-caused proliferation leads to the release of a C-terminal cysteine-rich cell membrane domain toward the ECM (Brandau et al. 2001).

Previous studies revealed an anti-angiogenesis effect of this extracellular molecular part, the C-terminal cysteine-rich domain. In an in vitro assay, the TNMD overexpression in human umbilical vein endothelial cells restrained cell proliferation and tube formation (Oshima et al. 2003). Moreover, this molecule is believed to function in lipometabolism regulation (Senol-Cosar et al. 2016; Tolppanen et al. 2008).

In the collagenous extracellular matrix of specific tendon tissue, e.g., the Achilles tendon and chordae tendineae cordis, 16 kDa cleaved C-terminal part of TNMD were found (Docheva et al. 2005; Kimura et al. 2008; Wilkins and Bisson 2012).

Due to the finding that a lack of TNMD causes premature tendon aging, which is distinguished with dysregulated Collagen fibrillogenesis and reduced cell density and proliferation, the supporting functions of TNMD in tissue maintenance were evidenced (Wilkins and Bisson 2012). TNMD promotes actions in which the C-terminal cysteine-rich domain alone is sufficient, like maintaining self-renewal and inhibiting senescence, and thus affects tendon-derived stem/progenitor cells positively (Bi et al. 2007). The time-dependent function of TNMD in tendon regeneration that was implied in initial studies needs to be examined in more detail (Tempfer et al. 2009; Kohler et al. 2013).

Analyzing the upstream regulators of TNMD expression was described with the help of the SCX knockout mouse line. Since the deletion of SCX caused the complete elimination of the TNMD expression, these results implied that SCX can directly drive TNMD transcription (Pennisi 2002), (Maffulli et al. 1998), (Sun et al. 2006).

In mice, a collateral decline of SCX and TNMD mRNA levels was caused by myostatin deletion (Kannus 2000), whereas myostatin stimulation caused their upregulation in fibroblasts, which implies that in the TNMD pathway myostatin is an upstream factor (Kannus 2000).

It would be of interest to further examine whether EGR-1 or EGR-2 also can affect TNMD expression, because EGR-1/EGR-2 transcription factors are capable of triggering SCX and Collagen I gene expression (Kielty et al. 2002).

A significantly lowered TNMD, Collagen I and fibromodulin expression was caused by a lack of the MKX gene (Butler et al. 1978). While SCX expression remained at the same level, a significant TNMD deficit could be found in MKX knockouts at E16.5 – implying that TNMD expression may be directly influenced by MKX. In BM-MSCs a TNMD upregulation was stimulated by the activation of the Wnt/β-catenin signaling pathway, while it had no effect on SCX and MKX expression. This result leads to the conclusion that the Wnt/β-catenin signaling does not depend on these transcription factors (Chuen et al. 2004). On the basis of the demonstrated abnormal collagen fibrillogenesis, which engenders stronger fibers pathologically, and concerning downstream factors, the TNMD knockout mouse model implied a coherence with Collagen I (Wilkins and Bisson 2012).

In mutant tendons, the lower proliferation and density of cells, the limited self-renewal, and earlier senescence of tendon stem/progenitor cells lacking TNMD coincided with an upregulation of the senescent marker p53 and downregulation of the proliferative marker cyclin D1 (Bi et al. 2007). A research that examined human heartstring ruptures reported a loss of TNMD expression at the lesion site as well as VEGF-A and MMP-1, MMP-2, and MMP-13 upregulation (Bishay and Gallo 2013).

The generation of tetracycline (Tet)-driven conditional TNMD overexpressing mouse model provided a method for in vitro comparison of tenogenic differentiation potentials to BM-MSCs, ASCs, differentiation factors, and tenocytes (Shi et al. 2017). The overall outcome was that a conditional TNMD overexpression promoted cell proliferation in BM-MSCs, ASCs, and tenocytes, in contrast to differentiation factors. Moreover, these findings demonstrated that BM-MSCs might have the highest tenogenic potential compared to the other three cell types. The tenogenic markers utilized in this study for the examination of this specific lineage differentiation were SCX, Collagens I, III, and VI, DCN, and TN-C. Tenocytes showed the slightest ability to induce tenogenic gene expression. Furthermore, in both BM-MSCs and ASCs, the overexpression of TNMD substantially restrained the differentiation toward osteogenic and chondrogenic lineages, while ASCs did not show any inhibition of adipogenic differentiation.

2.3.2 Neonatal Versus Adult Tenocyte Proliferation After Injuries

Lineage tracing in the neonatal injury model revealed that the progeny of primary tendon stubs and tenocyte proliferation trigger tendon regeneration. On the contrary, post-lesion, adult tenocytes are not subject to recruitment and remain quiescent; with lacking tenogenic cells, the adult tendon fibrotic regeneration is mediated by αSMA-expressing cells that continue to form a permanent scar. Godwin et al. (2013) hypothesized that, due to the mitotic activity of quite immature neonatal tenocytes, their inner potential differs much from adult tenocytes, because these adult cells are postmitotic. While key events like cell specification, differentiation, and patterning take place during the embryonic phase, at this stage, it may be difficult to distinguish between formation and regeneration, due to the abundant process of tissue growth (Godwin et al. 2013; Godwin and Rosenthal 2014). Moreover, various developmental aspects of tissue generation recur during the regenerative phase. Examining regenerative organisms, like adult salamanders, or tissues, like the bone, it can be inferred that certain events in the healing process are repeated developmental steps (Huang et al. 2013, 2015). In contrast to cell development, the adult surrounding inflammatory environment has crucial influence, and the lineage potential of tissue regeneration triggering cells is limited (Godwin et al. 2013; Godwin and Rosenthal 2014; Huang et al. 2013, 2015; Rinkevich et al. 2011; Roensch et al. 2013; Rux et al. 2016).

As the analysis of certain traumatic or neurogenic injuries and congenital diseases revealed, the heterotopic endochondral ossification can also be caused by inflammation (Kaplan et al. 2011; Potter et al. 2007). Furthermore, one of the roles of the tendon transcription factor MKX could be to hinder chondrogenic differentiation in tenocytes, as proven in a recent study (Suzuki et al. 2016). It can be assumed that the downregulation of the expression of MKX in adult tendons post-injury enables the differentiation of cartilage to the disadvantage of tenogenesis.

3 Gene Therapy

Recent developments in the field of genome editing will undoubtedly have a potentially large impact on current approaches to tendon therapy. One particularly striking example in this sense would be the CRISPR/Cas9 method (Chen and Qi 2017). This method allows for the activation or inactivation of any gene or group of genes chosen at will. A gene-specific guide RNA would be synthesized and then assembled in vitro with the Cas9 activator (or inhibitor) protein. This complex could then be transfected to the tissue.

Endogenous activation (or inhibition) of the chosen genes (e.g., XXX, YYY, and/or ZZZ) would ensue, and this would effect a stimulation of tenocytes conductive to tendon repair. It is important to emphasize here that, since we would be transfecting RNA and protein but, crucially, no DNA, there would not be any genome integration, i.e., no change in the genome would happen. This means that any change in gene expression induced by this approach would be temporary (not permanent), i.e., perfectly suited to the therapeutic objective.

An additional question would be the "in situ" transfection of the RNA-protein complex onto the affected tendon. One advance in this field seems particularly appropriate to mention here. Currently, cloned constructs are usually mixed with chemical reagents designed to facilitate entrance in the cell through the plasma membrane. The potential interference of such reagents (similar to detergents) with the therapy is not known. A novel approach, transfection through electroporation, could circumvent this problem. This procedure uses very short electrical pulses (high voltage/low current) in order to "open up" the cell membrane for a few milliseconds, allowing for delivery of the constructs into the cytoplasm. This is done with the help of electroporation for in vivo application (Adam et al. 2014). One can imagine that, in the near future, the electroporation of specifically designed CRISPR/Cas9 constructs on wounded tendons could become a therapy of election.

4 Outline for a Future Therapeutic Approach for Tendon Injuries

More sophisticated cell-based and/or pharmacological treatment methods for tendon regeneration will be applicable and developed in the future due to a deeper understanding of the molecular mechanisms of tendon development and healing and to hinder defect repair by scarring. At present, the traditional accomplishments in tendon regeneration are more or less suboptimal and thus could be improved with biological augmentation or intervention during the tendon healing process. A better understanding of the complex cellular environments during tendon healing phases could promote the development of new successful therapies.

In dependence on the different stages of tendon healing, it seems to be essential to avoid the cellular programming toward the mechanism of cellular repair and scarring and to substitute and augment the cellular tenocyte fraction to enable tendon regeneration. This might also include the necessity of new biological scaffolds aligned with the injured tendon fibers or other strategies for a better arrangement of the transplanted cells at the lesion site.

During the initial inflammatory phase of wound healing, the TGFß-1/CTGF cascade initiates fibroblast activation, and the presence of IL-1ß modulates the blocking of tenocyte differentiation. This implies that during the adult inflammatory tendon healing stage, the normal function of local or transplanted tendon progenitor cells might profit from TGFß-1 and IL-1β inhibition.

In this context the timing of administration of the biograft interleukin-1-receptor-antagonist protein processing system (IRAP) (Geburek et al. 2015), functional TGFß-1 antibody, and nonsteroidal and other anti-inflammatory drugs after tendon injury should be considered.

The immunomodulatory functions of MSCs in the inflammatory phase could be used to induce M2 macrophages in the healing site to suppress inflammation, suppress the proliferation of fibroblasts, and thus provide for regenerative tissue modeling. The anti-inflammatory therapeutic function of MSCs could be enhanced by priming before cell transplantation.

Another therapeutic function of MSCs includes the inhibition of the proliferative and pro-fibrotic phenotype of scarring through paracrine signaling.

According to the proteomic analysis, most of the GFs in tendon development and healing are secreted by AT-MSCs. GFs which are not expressed by AT-MSCs include BMPs and EGF, which might be key candidates for additional pharmaceutical substitution in relevant phases of tendon healing.

Different cell therapeutic sources could be used for the transplantation of tenocytes in the lesion site, ranging from TSCs to tendon-like cells directively differentiated from iPSCs or MSCs by transcription factor-mediated transdifferentiation. Tenocytes could be taken by small biopsies from native tendons, but it is not an obvious advantage in comparison to optimal differentiable stem cells in different stages with a high proliferation capacity.

Here the conditional activation of tendon-specific transcription factors in vitro might come into focus, and new elegant methods for overexpression without using viruses might come into play. Alternatively, these key transcription factors could also be activated by gene transfer or small molecules in the healing site to transdifferentiate the proliferating fibroblasts into a tenocyte phenotype.

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Stem Cell Therapy and Type 1 Diabetes Mellitus: Treatment Strategies and Future Perspectives

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Abstract

Type 1 diabetes mellitus (T1DM) is classified as an autoimmune disease which progressively results in the depletion of insulin-secreting β-cells. Consequently, the insulin secretion stops leading to hyperglycemic situations within the body. Under severe conditions, it also causes multi-organ diabetes-associated dysfunctionalities notably hypercoagulability, neuropathy, nephropathy, retinopathy, and sometimes organ failures. The prevalence of this disease has been noticed about 3% that has highlighted the serious concerns for healthcare professionals around the globe. For the treatment of this disease, the cell therapy is considered as an important therapeutic approach for the replacement of damaged β-cells. However, the development of autoantibodies unfortunately reduces their effectiveness with the passage of time and finally with the recurrence of diabetes mellitus. The development of new techniques for extraction and transplantation of islets failed to support this approach due to the issues related to major surgery and lifelong dependence on immunosuppression. For T1DM, such cells are supposed to produce, store, and supply insulin to maintain glucose homeostasis. The urgent need of much-anticipated substitute for insulin-secreting β-cells directed the researchers to focus on stem cells (SCs) to produce insulin-secreting β-cells. For being more specific and targeted therapeutic approaches, SC-based strategies opened up the new horizons to cure T1DM. This cell-based therapy aimed to produce functional insulin-secreting β-cells to cure diabetes on forever basis. The intrinsic regenerative potential along with immunomodulatory abilities of SCs highlights the therapeutic potential of SC-based strategies. In this article, we have comprehensively highlighted the role of SCs to treat diabetes mellitus.

Keywords

Autoimmune diseases · Diabetes mellitus · Pancreatic islets · Regenerative medicines · Stem cell therapy

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Abbreviations

CPPs	Cell-penetrating peptides
ESCs	Embryonic stem cells
HSCs	Hematopoietic stem cells
HSV	Herpes simplex virus
iPSCs	Induced pluripotent stem cells
ISC	Insulin-secreting cells
MSCs	Mesenchymal stromal cells
PTDs	Protein transduction domains
r-ATG	Rabbit anti-thymoglobulin
SCs	Stem cells
SCT	Stem cell therapy
T1DM	Type 1 diabetes mellitus
TAT	Trans-activator of transcription
UCB	Umbilical cord blood
WHO	World Health Organization

1 Introduction

A chronic condition of diabetes mellitus (DM) in which the body's pancreas fails to produce enough insulin to metabolize the excess amount of glucose is recognized as Type 1 diabetes mellitus (T1DM) or insulin-dependent diabetes. During this illness, the insulin-secreting β-cells are progressively destroyed due to a multifactorial genetic, idiopathic, or autoimmune disorder of the pancreas. Mostly, this chronic disease occurs in the early years of childhood, but its prevalence has also been observed in the adults of 30–40 years of age. T1DM occurs predominantly in males as compared to that of females (Harjutsalo et al. 2008; Ostman et al. 2008). Several factors notably microbial infections, environmental pollutants, and seasons of winter and autumn happen to trigger this natural autoimmune disorder (Kukko et al. 2005; Yeung et al. 2011). In general public, its occurrence is about 0.5% with an alarming annual increment of 3% that has highlighted the serious concerns for healthcare professionals around the globe. The typical symptoms of T1DM include polyphagia, polyuria, and polydipsia with hyperglycemia. Eventually, it results in multi-organ dysfunctions like nephropathy, neuropathy, ketoacidosis, hypercoagulability, diabetic retinopathy, and cardiovascular diseases. The extreme conditions could even lead to organ failure (Thakkar et al. 2015; Vanikar et al. 2016; Yoon and Jun 2005; Zimmet et al. 2001).

World Health Organization (WHO) and American diabetes association has classified T1DM as an immune-mediated and idiopathic disease. In diabetic patients, the glycosylated hemoglobin and blood sugar levels help to determine the diabetic status while low levels of serum C-peptide act as highly acceptable indicator for T1DM diagnosis. Individuals with markers of autoimmunity are antibodies to β-cells, islets cells, glutamic acid decarboxylase, protein tyrosine phosphate like ICA 512 or 1a2, DQ8, and HLA-DR3/4 genotypes are seriously prone to the development of T1DM (American Diabetes 2010; Czaja 2016).

During the histopathology of islets, insulitis is observed at an earlier stage while irreversible depletion of β-cell destruction, fibrosis, and atrophy represent later stages. The prevalence of T1DM around the globe and its worse effect on the quality of life has increased the interest of the medical practitioners to find alternative strategies to cure this horrible foe (Czaja 2014; Greenbaum and Atkinson 2011; Manns and Strassburg 2011). Under the current scenario of treatment options for T1DM, new treatment goals do include the prevention of production of C-peptide and to block or control the autoimmune depletion of β-cells. In this connection, a few notable developments have been made. In adults with T1DM, the stimulated levels of C-peptide concentration got stable as a result of synthetic immunomodulator "DiaPep277" administration at 3 months interval, and a decline was observed in 1 year time period (Buzzetti et al. 2011; Schloot et al. 2007). However, this synthetic immunomodulator was unsuccessful to provide much-anticipated long-lasting effects. In children with newly diagnosed T1DM, the anti-CD3 antibodies in phase 3 trials, the Diamyd vaccine was unable to meet the primary end points (Walter et al. 2009, 2011). Fusion protein CTLA4-immunoglobulin (ig; abatacept) when administered intravenously for 2 years managed

to prevent the stimulated C-peptide concentration for a maximum period of 9 months only (Orban et al. 2011). From these results one could conclude that single-agent immunosuppression could not control or block the autoimmune depletion of β-cells. So, more specifically targeted therapeutic strategies are required to meet new treatment goals that have been briefly discussed in this article.

2 Insulin Secretion and Glucose Homeostasis

Insulin is produced at a rate of approximately 2 μmol/Kg/min under fasting conditions, and an increment of about five- to tenfold is observed after meals (Eaton et al. 1980). Regulation of secretion of insulin by the β-cells of the pancreas is required for the glucose homeostasis within the body (Dube et al. 2013). This is accomplished by an adequate number of normally working β-cells commonly referred as β-cell mass. Normally human pancreas contains one million islets, each containing at least 2000 β-cells (Rahier et al. 1983; Stefan et al. 1982). Collectively, β-cells do create about 1.5% of the total pancreatic mass (Bonner-Weir et al. 1993). A single β-cell of a size of 15 μm stores about 10,000 insulin granules. At least 200,000 crystalline molecule of insulin could be found on a single insulin granule of about 300 nm. Initially, glucose intake at the cell membrane triggers this well-organized and coordinated process which finally concludes with the secretions of insulin (Ball and Barber 2003; Halban 2004). For a consistent smooth endogenous insulin production, the autoimmune depletion of β-cells should be avoided to keep them functionally viable as their generation has become a challenging task.

Under fasting state, the blood sugar level is normally supposed to be maintained between 70 and 126 mg/dl. Postprandial blood sugar should be 110–140 mg/dl measured 2 h after meals. If random blood sugar exceeds 200 mg/dl, it indicates the development of DM. For further confirmation in such individuals, oral glucose tolerance test is performed to confirm blood sugar of ≥200 mg/dl after 2 h of taking 75 g glucose orally and HbA1c ≥ 6.5 which confirms the occurrence of DM. The low levels of serum C-peptide helps to differentiate T1DM from T2DM. In 1981, children newly diagnosed with T1DM were treated with prednisone to block autoimmune destruction of pancreatic β-cell, and an improvement in urinary C-peptide was seen for 1 year (Couri and Voltarelli 2009; Elliott et al. 1981). Afterward cyclosporine, azathioprine, plus prednisone and azathioprine were used in further different trials. These trials resulted in positive responses on the maintenance of C-peptide level and on the control of autoimmune β-cell depletion. However, these interventions did not gain popularity due to toxicity complains. Consequently, the attentions were diverted to develop some novel and alternative approaches.

3 Pancreas and Islet Transplantation

In 1966, Kelly and colleagues were the first to execute pancreatic transplantation to treat T1DM. Subsequently, more than 25,000 patients have received pancreatic transplantations around the world (Gruessner and Sutherland 2005; Jahansouz et al. 2011). In successful cases, the hyperglycemic conditions were controlled rapidly without the use of external insulin. However, high morbidity rates due to surgery and lifelong dependency on side effects producing immunosuppression were the major drawbacks. In few cases, the side effects resulted in the recurrence of diabetes (Cefalu 2012). Later techniques were developed for the extraction and transplantation of islets to cure T1DM (Fotino et al. 2015; Ramirez-Dominguez 2016; Ricordi et al. 1989). However, these efforts did not help patients to survive for more than 5 years under insulin-free conditions. Then the focus was turned to the encapsulation of immune modulation and the development of new delivery techniques; however, no worth mentioning success has been achieved yet (Gibly et al. 2011; Ryan et al. 2005).

4 Stem Cell Therapy: An Alternative Approach to Islet/Pancreas Transplantation

In order to find a substitute for insulin-secreting β-cells, the researcher focused to explore the suitability and potential of stem or progenitor cells to produce mass of insulin-secreting cells (ISC). The cell-based therapies were aimed to downregulate the immune system and to halt or abolish the autoimmune depletion of these cells. The stem cell generation and their differentiation into normally functioning insulin-secreting β-cells was focused to treat T1DM. The stem cell therapy (SCT) is meant to improve the quality of life by achieving a normalized glycemic control, reducing side effects of synthetic immunosuppression and preventing diabetic complications. Although the restoration of C-peptide secretions and an insulin independence are primarily desirable goals, SCT only manages to reduce the insulin requirements significantly (Santana et al. 2006).

SCT is considered to generate a desired cell/tissue by the differentiation process under strict proliferating and reproducibility control without disturbing the surrounding tissues. It helps to render a quality life to the recipient by immunological resetting (Bianco and Robey 2001). This unique process is supposed to be nonirritant. These cells should manage to produce, store, and secrete insulin under glycemic conditions to avoid the development of hyperinsulinemic hypoglycemia from the induction of ISC.

4.1 Stem Cell Therapy for β-Cell Regeneration

The immunomodulatory capacity and intrinsic regenerative potential of stem cells have highlighted the significance of therapeutic role of stem cell-based strategies. These SC-based strategies help in the restoration of immune and glycol-metabolic homeostasis. For the purpose of transplantation, a supply of self-sustaining glucose responsive ISCs could be made available by harnessing the regenerative capacity of SCs. The immunomodulatory potential is exploited for the preservation of residual β-cell mass by avoiding β-cell destruction and for the facilitative regeneration of endogenous β-cells. Further, the immunomodulatory properties help to prevent the recurrence of autoimmunity and also minimize the chances of innate/alloimmune graft rejection (Barcala Tabarrozzi et al. 2013; Fiorina et al. 2011). The stem cells with potent immunomodulatory properties alone and in combination with β-cell replacement strategies have been found useful to reverse the hyperglycemic conditions in patients of T1DM (Jurewicz et al. 2010; Rackham et al. 2011). SCs from various sources have been tested for their potential to restore immune homeostasis and their ability to regenerate β-cells. They have also been tested for their ability to promote longitudinal graft survival. These include adipose tissue-derived (AD) mesenchymal stromal cells (MSCs), umbilical cord blood (UCB)-derived MSCs, bone marrow (BM)-derived hematopoietic stem cells (HSCs), pancreas-derived multipotent precursor cells, induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs). The pancreatic β-cell progenitors residing in exocrine tissue, islet proper and ductal epithelium, facultative β-cell progenitors from endometrium, liver, and spleen have also been tested for aforementioned properties.

4.2 Role of Embryonic Stem Cells in Diabetes

The ESCs were considered as the best source to generate pancreatic islet cells because of their ability of differentiation into any cell line in proper time span and with appropriate signals. In 2000, Soria and colleagues successfully generated islet cells by genetic modification of mouse ESCs. They also managed to demonstrate a few month lasting improvements in hyperglycemia (Martin 1981; Soria et al. 2000). Only the cells which were activating the insulin promoters survived when the ESCs were grown in the presence of antibiotic. The cells were cultured and cloned under various conditions. With low

concentration of glucose, the cultured cells were differentiated into glucose-responsive cells, and the insulin secretion was observed to be about sevenfolds. However, the cell selection before full differentiation and the presence of non-islet ISCs restricted the creation of insulin-positive cells (Assady et al. 2001; Hori et al. 2005; Segev et al. 2004). Lumelsky and co-workers conducted experiments on mouse ESCs and managed their differentiation into insulin-producing cells which were similar to pancreatic islets (Lumelsky et al. 2001). The mouse ESCs from endoderm were also used to develop pancreas (D'Amour et al. 2006). It was also reported that ESCs can also express insulin gene. In other experiments, the insulin-secreting β-cells were characterized as 1–3% only in embryoid bodies. In this case, the release of C-peptide was observed in response to stimuli like cyclic adenosine monophosphate and potassium chloride while no insulin secretion was observed in response to glucose. In other experiments to get more precise outcomes, the in vitro differentiation was blocked when the cells were resembling to 6–9-week-old embryos. These differentiated cells were transplanted into epididymal fat pad in immune-deficient mice, and glucose-dependent insulin secretion was measured. The C-peptide secretion was low at 1-month post transplantation point; however, in 3-month time, the secretion level was as high as it could be found with the transplantation of 3000–5000 human islets (Assady et al. 2001). The risks of teratogenicity and some religious and ethical issues related to the use of ESCs diverted the attentions to search for new alternatives (Liu et al. 2013; Shih et al. 2007; Werbowetski-Ogilvie et al. 2009).

4.3 Adult Pancreatic Stem Cells

The islet consists of four types of functionally potent cells including insulin-producing β-cells, glucagon-producing α-cells, pancreatic polypeptide-producing cells, and somatostatin-producing δ-cells. Due to the clonogenic potential and multipotent characteristics, the adult pancreatic SCs can act as a potent source of pancreatic β-cells. The pancreas has been believed to be the main source of β-cells for the regeneration of tissues. This proposal has been supported by the proliferation and differentiation characteristics of ductal cells and the pancreatic duct ligation model of injury. In adult mice, the β-cells mass was noted when the duct lining neurogenin-3 (NGN3 +) endocrine precursors get activated (Ramiya et al. 2000; Wang et al. 1995; Xu et al. 2008). Advance and innovative strategies are required to employ for proper growth, appropriate isolation, and differentiation into β-cells. The functional islets were produced when epithelial cells of pancreatic duct were harvested and induced in vitro (Seaberg et al. 2004). In an adult mouse, reports demonstrated appreciable clonal potential and characteristics of multiple progenitor cells of pancreas, and the produced β-cells showed glucose-dependent insulin secretions. The challenges of inducing β-cell differentiation without genetic mutations and the issues related to harvesting, purification, and growth of various types of pancreatic progenitor cells have forced the researchers to set new future targets.

4.4 Adult Non-pancreatic Stem Cells

The dead or damaged cells and tissues could be replaced by MSCs and HSCs as they possess multipotency of proliferation. The detail has been described in the proceeding sections.

4.5 Mesenchymal Stromal Cells

Friedenstein and group were the first to identify the MSCs from rat BM (Friedenstein et al. 1966). One could harvest MSCs from BM, adipose tissues, or other organs. MSCs obtained from various sources including the umbilical cord, adipose tissues, and BM were found similar in isolation, morphology, colony frequency, immune response, and differentiation capacity. The AD-MSCs and BM-MSCs were found equally potent when compared for their functional capacity. They were used in diabetic rats to alleviate doxorubicin-induced cardiac dysfunctions by

promoting the process of angiogenesis, reducing the collagen deposition and infiltration of immune cells. Both in in vivo and in vitro tests, immunomodulatory properties have been displayed by MSCs (Hoogduijn et al. 2010; Masoud et al. 2012). The minimum criteria to define human MSCs have been proposed by Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. They should display the characteristics of plastic adherence under standard culture conditions and also display adipogenic, chondrogenic, and osteogenic differentiation capacity; they should express CD73, CD90, and CD105 on their surface and must lack expressions of hematopoietic lineage markers c-kit CD11b, CD14, CD19, CD34, CD45, CD79α, and human leukocyte antigen (HLA)-DR (Dominici et al. 2006; Trivedi and Vanikar 2013). The adipose tissue has become the easy and most acceptable source of isolation and generation of MSCs. The growth of MSCs is carried out in an extract of regenerative factors subsequently; they get transformed to glucose-responsive insulin-secreting islet like clusters (Oh et al. 2004). Under specific conditions, insulin-secreting SCs have also been reported from BM-MSCs (Fiorina et al. 2009; Tariq et al. 2013; Urban et al. 2008). MSCs alone or in combination with HSCs display positive effects on the glycemic control in animal models of T1DM. With streptozotocin (STZ)/radiation-induced injury, BM of the mice was injected with combination of HSCs and MSCs to observe normalization of blood glucose. The proliferation of pancreatic β-cells-specific cells could be inhibited by MSCs and thus halt the autoimmune depletion of pancreas. BM-SCs are capable of stimulating the regenerative process of damaged pancreatic β-cells. The BM-MSCs differentiated into insulin-secreting β-cells were used for the treatment of STZ-induced diabetes in rats. Very encouraging and satisfying results were seen in the form of reversal of diabetic parameters, left ventricular contractility, returning of normal weight, and cardiac parameters like heart rate. The high fibrinogen level, reactive oxygen species, basal Ca^{+2} levels, improved systolic blood pressure, and decreased levels of anti-apoptotic marker Bc1–2 demonstrated endothelial activations (Haidara et al. 2015).

4.6 Insulin-Secreting Cells from SCs

In 2007, Karnieli et al. and Li et al. demonstrated that functional ISCs were differentiated from human BM-MSC gene pancreatic duodenal homebox-1 (Karnieli et al. 2007; Li et al. 2007). In appropriate in vitro conditions, the BM-MSCs from diabetic patients were made to differentiate into ISCs by Sun and research group (Sun et al. 2007). The ISCs were generated successfully from autologous ADMSCs generated in vitro in T1DM patients and these cells managed to express paired box gene 6 (Pax-6). This gene is a key regulator for normal islet cell development and upregulates the insulin and islet promoter factor-1 and insulin expression and transcription factor for β-cell maturation and normal pancreatic development (Trivedi et al. 2008).

4.7 Induced Pluripotent Stem Cells

The induced pluripotency is referred to a process of PSC formation from non-pluripotent cells. Under specific conditions, the transformation of somatic cells into PSCs is termed as iPSCs. This remarkable discovery led to the outbreak of the research for reprogramming of the cells (Geoghegan and Byrnes 2008). The iPSCs displayed similarities with ESCs as they possess high telomerase activity. The gene promoters are also hypo-methylated. Reprogramming of human somatic cells like skin fibroblasts could produce human iPSCs. Consequently, they could be a better option to human ESCs, thus eliminating the related religious or ethical issues. However, they could pose severe threats of tumor formation, and also there are the chances of their rejec-

tion due to autoimmunity in T1DM (Okita et al. 2013; Takahashi and Yamanaka 2006).

5 Protein Transduction Technology for Induction of Insulin-Producing Cell from SCs

Various peptides called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) developed through protein transduction technology have shown characteristic abilities to translocate in live cells (Noguchi and Matsumoto 2006). These peptides could be crosslinked to PTDs covalently or internalize into the cell directly when produced as recombinant fusion proteins. Both in in vivo and in vitro studies, the biologically potent peptides have been delivered to cells. The list of mostly studied PTDs includes homeodomain transcription factors like herpes simplex virus (HSV) type-1, antennapedia, and HIV-1 trans-activator of transcription (TAT) proteins and VP22. Mechanistically, PTD-mediated protein transduction is executed through endocytosis followed by the route from the vesicle into the cytoplasm (Noguchi et al. 2003, 2005). Due to a facilitating role of PTDs in the differentiation of SCs into iPSCs, this technology is expected to dominate all other therapeutic approaches for diabetes. The PTD sequence has also been observed in the structure of two pancreatic endocrine transcription factors called PDX-1 and neuroD/β2 proteins. In pancreatic ductal progenitor cells, the β2/neuroD or PDX-1 managed to induce insulin expression. Domínguez-Bendala and colleagues in an in vitro studies demonstrated that the pancreatic endocrine differentiation was stimulated by TAT-mediated NGN3 protein transduction (Dominguez-Bendala et al. 2005). It is suggested that the third helix of homeodomain of transcription factor Isl-1 manages to internalize into the cells (Kilk et al. 2001). So, PTDs serve as a novel strategy to deliver exogenous transcription factors like Isl-1, NGN3, neuroD/β2, PDX-1, etc. Without the use of gene transfer technology, the PTDs could be used for generating ISCs from progenitor/stem cells. The MSCs have become vehicles for this approach due to the required characteristics and potentials.

6 Stem Cell Therapy in T1DM: Clinical Experience

In 2007–2008, 23 T1DM patients of age group 13–31 years were infused with BM-mobilized HSCs in peripheral circulation for 6 weeks. The HbA1c level of 8.4% and average blood sugar level 395.6 mg/dL was noted at the time of diagnosis (Voltarelli et al. 2007, 2008). In this protocol, heavy conditioning of rabbit anti-thymoglobulin (r-ATG) and cyclophosphamide was used. Almost all subjects displayed improvements after SCT; most of them remained insulin-free for a short time while only 8 patients were found insulin-free for 5–6 months. The same protocol was followed in recently diagnosed T1DM patients at the University of Nanjing. In 16 patients, half of the cells were injected in the pancreas and half in periphery. A short-term follow-up confirmed a decrease in insulin requirements, two patients quickly became insulin-free, and the insulin requirements dropped to 50% in remaining. The patient suffered from diabetes for a longer time in this series. No significant improvements were reported when 15 patients of T1DM were treated with autologous mononuclear cord blood cells at Florida University (Haller et al. 2009). In 2008, Trivedi et al. reported a novel therapy for T1DM with much effective response (H. L. Trivedi et al. 2008). This therapeutic approach was different from the others in different aspects. The first difference was the generation of insulin-secreting AD-MSCs from insulinopenic patients. The second was the generation of BM-HSCs from the same set of patients. Thirdly, there was no use of exogenous material. The forth difference included the infusion of cells into subcutaneous tissue and thymic and portal circulation. The insulin requirements dropped down to 30–50% while serum C-peptide levels increased

from 4- to 26-folds. These effects sustained for 3 years without the use of any immunosuppression. The grafting cells were conditioned somewhat similarly as others reported in their studies; however, subtotal lymphoid irradiations were used additionally. No graft-versus-host disease or ineffective incidents were reported. Eleven patients of T1DM with 1–24 years of disease were infused with AD-MSC-ISCs with BMHSCs by Vanikar and colleagues (Vanikar et al. 2010). In all patients, a mean decrease in requirements of exogenous insulin was from 1.1 units/kg BW/d to 0.63 units/kg BW/d, C-peptide levels increased from 0.1 ng/mL to 0.38 ng/mL, a drop in Hb1Ac from 8.47 to 7.39%, and all patients became free of diabetic ketoacidosis events. In 20 T1DM patients, a randomized open-labelled trial was carried out, 10 with allogeneic SCT and 10 with autologous SCT (Thakkar et al. 2015). Both groups were infused with BM-HSCs and AD-MSC-ISCs. Exactly identical treatments were received by both groups into liver via intraportal route, subcutaneous fat pad, and thymic circulation. To take advantages of better grafting and tolerogenicity of the liver, the portal circulation was carried out while thymic infusion was selected to attain central tolerance (Sprent and Kishimoto 2001; Starzl 2001). A part of the cells was kept in abdominal tissue, taking it as immunologically privileged location, to really serve as a backup reservoir for uninterrupted insulin supply (Prokhorova et al. 2009). The results for the control of hyperglycemia were better in autologous arm compared to allogenic. This research also confirms that the patients of T1DM could use their own adipose tissue reserves for treating their disease without the help of any external donor (Table 1).

7 Future Strategies to Treat T1DM

Due to multifaceted autoimmune activities, T1DM has established itself as a complex disease as it is evident from a number of studies involving multiple trials on animals, rodents, and humans. So, a therapeutic approach is also supposed to be a multifaceted based on multipronged strategy (Concannon et al. 2009; Pozzilli 2012; Staeva et al. 2013). The long-lasting effects are achieved by compulsory use of ISCs; however, the expectations for a complete cure of T1DM have not been achieved yet. The inadequacy of infused SC dose and reactivation of autoimmune process could be categorized as the main drawbacks. Further, few questions could include how many cells should be there? If infusion is required to be repeated, what will be the best time interval, a new site, and or method of grafting cells be considered alternatively? The MSCs have proved as the best available option by inducing significant immune tolerance against autoimmunity through the generation and recruitment of regulatory cells. In conclusion, SCT is an innocuous and economical measure among the currently available treatment options for curing T1DM.

SCT is a relatively new therapeutic approach which is able to control the minute-to-minute fluctuations in systemic blood glucose, and this therapy is more effective at early stages after the diagnosis of diabetes mellitus than intervention at later stages. Stem cell transplantation can represent a safe and effective treatment for selected patients of diabetes mellitus. In spite of this, some hurdle and issues also exist which need to be addressed such as lack of donors, safety concerns, low success rates of islet isolation, and requirement for lifelong immune suppression (long-term recovery remains to be addressed). An average weight person requires transplantation of roughly 340–750 million islet cells to effectively resolve the disease. However in clinical practice, this demand can be fulfilled by two or three donors for a single diabetic patient. There are several crucial challenges that must be needed to address and further investigations required to establish stem cell-based therapies for the potential treatment and cure of diabetes. In this chapter we will critically focus on the different stem cell delivery strategies which are being used for treatment of diabetes mellitus.

Conflict of Interest Nothing to declare.

Table 1 Summary of different stem cell strategies and the main goals achieved at clinical or preclinical level

Sr. #	Treatment strategy	Outcomes	Study design	References
1	In 20 T1DM patients, open randomized trial: 10 autologous SCT, 10 allogenic	Results showed the superiority of autologous over allogeneic	Clinical	Thakkar et al. (2015)
2	The rats with STZ-induced diabetes were treated with BM MSCs differentiated into insulin-secreting β-cells	Reversal of cardiac parameters like left ventricular contractility, heart rate, and diabetic parameters; weight returned to the normal. The high fibrinogen levels, improvements in systolic BP and decrease in anti-apoptotic marker Bc1–2, reactive oxygen species, and basal calcium ion levels represented the endothelial activation	Experimental	Haidara et al. (2015)
3	11 patients, same as above	Decreased mean exogenous insulin requirement from 1.14 U/kg BW/d to 0.63 U/kg BW/d, Hb1Ac decreased from 8.47 to 7.39% and serum C-peptide levels increased from 0.1 ng/mL to 0.38 ng/mL. No DKA episodes were seen in all patients	Clinical	Vanikar et al. (2010)
4	During this study, 15 patients were treated with autologous mononuclear cord blood cells	No improvements were seen in any patient	Clinical	Haller et al. (2009)
5	Under the conditioning of cyclophosphamide + r-ATG- DM <6 weeks old. BM-HSCs were infused in periphery of 23 patients	All patients reported subjective improvements while only 8 patients were found insulin-free for 5–6 months	Clinical	Voltarelli et al. (2007, 2008)
6	In vitro generation of BMHSC + autologous AD MSC-ISC and infusion in thymic + portal circulation and subcutaneous tissues under conditionings of cyclophosphamide + r-ATG, s-TLI (200 cGY × 5), r-ATG, 1.5 mg/kg BW in five patients	The effects were sustained for 5 years with 4- to 26-fold increase in serum C-peptide levels and 30–50% decrease in insulin requirement	Clinical	Trivedi et al. (2008)
7	In 16 patients, the BM-HSCs were injected half in the pancreas and half in periphery. H/O DM for a long time	Short-term follow-up displayed decrease in insulin requirements. Two patients became transiently free from insulin while the remaining had 50% decrease in insulin requirement	Clinical	Haller et al. (2009)
8	By genetic modifications, islet cells were generated from mouse ESCs	For a few months, improvements in hyperglycemia were noted	Experimental	Soria et al. (2000)

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Characterization of Senescence of Human Adipose-Derived Stem Cells After Long-Term Expansion

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Abstract

Introduction: Since the 1980s, adipose-derived stem cells (ASCs) have become a powerful and potential source for stem cell-based therapy, regenerative medicine, and even drug delivery in cancer treatment. The development of off-the-shelf mesenchymal stem cells (MSCs), including ASCs, has rapidly advanced in recent years with several clinical trials and approved products. In this technology, ASCs should be expanded long term in order to harvest higher cell number. In this study, senescence of ASCs after long-term expansion was evaluated. **Methods**: Human ASCs (hASCs) were isolated and cultured continuously at a density of 10^3 cells/cm^2 up to passage 15. The cells were assessed for aging via changes in the following: characteristics of MSCs, mitochondrial activity, accumulation of beta-galactosidase, and expression of tumor suppressor genes. **Results**: The results showed that following in vitro expansion to the 15th passage, ASCs did not show changes in immunophenotype, except for decreased expression of CD105. However, the cells increased in size and in shape and complexity (toward the "fried egg" morphology). They also almost ceased to proliferate in passage 15. Nonetheless, they maintained in vitro differentiation potential toward osteoblasts, chondrocytes, and adipocytes. Expression of tumor suppressor genes p53 and p16 did not significantly change, while p27 was significantly downregulated. Mitochondrial activities also decreased slightly in culture from passage 5 to passage 10 and remained stable to passage 15. ASCs also showed increased accumulation of beta-galactosidase in culture, but it was negligible. **Conclusion**: In conclusion, hASCs exhibited some particular characteristics of aged stem cells when the number of subculture cells increased. However, up to passage 10, ASCs also retained almost all of the characteristics of MSCs.

Keywords

Aging · Senescence · Long-term · In vitro culture · Mesenchymal stem cells · Adipose-derived stem cells

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Abbreviations

ASCs:	Adipose-derived stem cells
BM-MSCs:	Bone marrow-derived mesenchymal stem cells
CFU-F:	Fibroblast colony-forming units
DE:	Germany
FBS:	Fetal bovine serum
FCM:	Flow cytometry
FSC:	Forward scatter
hASCs:	Human adipose-derived stem cells
iPSCs:	Induced pluripotent stem cells
KR:	Korean
MSCs:	Mesenchymal stem cells
PBS:	Phosphate-buffered saline
ROS:	Reactive oxygen species
SA-β-gal:	Senescence-associated β-galactosidase enzyme
SSC:	Side scatter
SVF:	Stromal vascular fraction
UK:	United Kingdom
USA:	United States

1 Introduction

Adipose-derived stem cells (ASCs) were discovered in 1980 by Plaas and Cryer (Plaas and Cryer 1980) and shared many characteristics with bone marrow-derived mesenchymal stem cells (BM-MSCs) (Mizuno et al. 2012). However, from the 2000s, studies on ASCs have accelerated (Salgado et al. 2010). ASCs are an ideal source of stem cells for research and therapeutic applications and have the ability to differentiate and trans-differentiate into many cell types belonging to the three germ layers (Rodriguez et al. 2004; Ugarte et al. 2003). In addition, the use of ASCs is not restricted by bioethics as embryonic stem cells (ESCs) or not subjected to technical constraints, as is the case with induced pluripotent stem cells (iPSCs) (Mizuno et al. 2012).

Moreover, adipose tissue is abundant, easily accessible, and usually removed in cosmetic liposuction (Bunnell et al. 2008). According to Laura Frese et al. (2016), subcutaneous adipose tissue may be approached repeatedly by minimally invasive methods. ASCs are also obtained by a relatively simple procedure, and they have age-independent quality and proliferation. In addition, ASCs can secrete many soluble factors that mediate important activities, such as mobilizing endogenous stem cells, supporting tissue regeneration, and engaging in immune modulation (Salgado et al. 2010; Hu et al. 2013). All of these make ASCs an ideal alternative source of BM-MSCs for the clinic (Frese et al. 2016).

Since ASCs have such great potential, research on ASCs and its applications have been steadily increasing. However, the number of cells obtained via liposuction is not high. Human ASCs account for only 2% of cells in adipose tissue or about 5,000 fibroblast colony-forming units (CFU-F) per 1 gram of fat (Strem et al. 2005). Stefan Lendeckel et al. (2004) obtained 2.95×10^8 mononuclear cells in 42.3 g of gluteal area; only about 2–3% of these cells were stem cells (about 5.9×10^6 cells) (Lendeckel et al. 2004). At the same time, 1-2 $\times 10^6$ cells are needed for grafting per 1 kg of body weight (Ikebe and Suzuki 2014), which corresponds to $60–120 \times 10^6$ cells per weighing 60 kg. Thus, the number of primary cells is insufficient for use in treatment or even research studies. Hence, subculture is necessary. However, multiple times of passaging, coupled with the effects of long-term in vitro culture, might cause these cells to age.

Aging (or senescence) is derived from *senex* in Latin, which refers to an extremely complex process that affects most of the body's biological functions, often leading to disease or even mortality from various stresses (Yu and Kang 2013; Campisi 2013). The cause of aging is the accumulation of cell damage or injury over time (Lopez-Otin et al. 2013). The aging tissue undergoes a progressive decline in homeostasis and regenerative capacity caused by degeneration of tissue-specific stem cells, stem cell niche, and stem cell signaling (Oh et al. 2014). Although the body becomes older, aging also plays a very important role in maintaining the integrity of the body when neoplastic clones appear (Lynch 2006).

In fact, aging and cancer are two different manifestations of the same process, accumulating in cell damage (Campisi 2013; Lopez-Otin et al. 2013; Adams et al. 2015). Damage to DNA can affect important genes and transcription pathways. Wagner et al. (2010) reported that genes involved in cell cycle, DNA replication, mitosis, and DNA repair were significantly reduced in late passages (Wagner et al. 2010). Cancer is related to the stem cells of tissue (Devereux et al. 1999; Sell 2010) through DNA damage in replication, telomere shortening, and epigenetic and metabolic alterations (Adams et al. 2015). There has been much evidence for abnormalities of chromosome in long-term cultured MSCs (Miura et al. 2006; Bochkov et al. 2007; Foudah et al. 2009). Conversely, it has been confirmed by many reports that MSCs are safe for clinical treatment despite undergoing long-term culture (Bernardo et al. 2007; Wang et al. 2013; Zhang et al. 2007; Choi et al. 2010; Zheng et al. 2009; Li et al. 2009).

Besides the DNA in the nucleus, mitochondrial DNA is also a vulnerable target (Linnane et al. 1989). There have been many studies which have shown that defects in mitochondrial DNA are almost due to errors in the early stages of development rather than to oxidative damage (Ameur et al. 2011). About 40 years ago, it was hypothesized that mitochondrial dysfunction could be the cause of aging due to the accumulation of damage caused by reactive oxygen species (ROS) (Passos and von Zglinicki 2005). In recent years, the understanding of mitochondrial aging from research has progressed tremendously. There is plenty of evidence that shows that increasing ROS concentrations not only causes aging but also extends the life of *C. elegans*, yeast, and mice; notably, addition of antioxidants does not help prolong life (Lopez-Otin et al. 2013). Thus, ROS are considered to be a survival signal that is triggered to compensate for damage caused by aging. However, it can become a double-edged sword in the attempt to maintain that survival.

In the survival process, the cells are constantly stressed and damaged by many internal and external factors. Depending on the level of stress and ability to respond, the cells can completely recover or succumb to cell death. Proliferating cells may respond by permanent cell cycle arrest, referred to as cellular or replicative senescence (Campisi and d'Adda di Fagagna 2007). Cellular senescence was described more than 50 years ago when Hayflick et al. pointed out that normal stem cells have limited division in culture (Hayflick and Moorhead 1961). Today, we know that the aging phenomenon described by Hayflick is caused by the shortening of telomeres. Telomere attrition, combined with rapid growth rate in culture, is involved in the gradual accumulation of aging cells (Wagner et al. 2008) and promotes the depletion of stem cells (Lopez-Otin et al. 2013). To limit the shortening of telomeres, certain types of cells have the presence of telomerase. This enzyme is active in cancer cells (Blasco 2005), germ cells (Bekaert et al. 2004), and embryonic stem cells (Hiyama and Hiyama 2007). In adult stem cells, including hASCs, telomerase activity is low or absent (Hiyama and Hiyama 2007; Baxter et al. 2004). However, even with telomerase, telomere defects remain invisible to the DNA repair system due to the presence of shelterin (Palm and de Lange 2008).

Understanding the molecular pathway involved in stem cell decay is important in developing targeted therapies for aging (Oh et al. 2014). So far, there have been no specific molecular markers for evaluating cellular aging (Wagner et al. 2010). Activity of senescence-associated β-galactosidase enzyme (SA-β-gal) in aging cells was not seen in quiescence (Dimri et al. 1995). SA-β-gal staining procedure, while seemingly easy, is difficult to quantify, as it is positively and exclusively expressed in almost all aging cells with the "fried egg" morphology. Anyway, it should be noted that use of SA-β-gal does not affect the aging process. Despite the quantitative limitations and potential for analysis, SA-β-gal is still the most widely used aging marker (Wagner et al. 2010). However, in order to accurately assess the level of aging of MSCs cultured in vitro, more specific molecular markers are required.

The next aging molecular markers are tumor suppressor genes. Aging is the price that the cells have to pay for their ability to inhibit tumor.

Activation of INK4/ARF that encodes both p16INK4a/Rb and p19ARF/p53proteins plays an important role in inhibiting the proliferation of damaged cells. However, INK4/ARF activation may aggravate the aging process when INK4/ARF is overexpressed (Lopez-Otin et al. 2013). The number of mechanisms that cause aging in response to oncogenic lesions is increasing, but the first pathways reported, p16INK4a/Rb and p19ARF/p53 pathways, are still the preeminent ones (Campisi 2013; Serrano et al. 1997; Pelicci 2004). Inactivation of p53 usually leads to the accumulation of mutant proteins, which occur during tumor formation (Rivlin et al. 2011). In addition, Wagner et al. (2010) reported that *p16ink4a*, which plays an important role in regulating the stemness of MSCs (Yu and Kang 2013), was upregulated in high passages (Wagner et al. 2010). Besides these tumor suppressor genes, P27 gene (an inhibitor of the cyclin-dependent kinase) not only plays an important role in regulating the cell cycle but also regulates other functions that depend on cell cycle, such as differentiation and migration (Gao et al. 2010).

According to Wagner et al. (2009), MSCs obtained from donors of different ages have almost no difference in immunophenotype and differentiation. In other words, cell culture is the major contributing factor to the MSC aging process. Indeed, the nonphysiological conditions of in vitro culture, such as cellular isolation, type of culture medium, and culturing method, might increase the risk of mutations, abnormal chromosomes, and even malignant transformation (Wagner et al. 2010). Through many passages, cells become aged and express SA-β-gal; the process is accompanied by a reduction in fat differentiation and an increase in bone differentiation. At the same time, the ability to proliferate is lost in about 50–90 days of culture (Wagner et al. 2009). This result is similar to a few other studies with some negligible differences (Maredziak et al. 2016; Ye et al. 2016; and Baer et al. 2010). In a study by Huang et al. (2010), trypsin used during cell proliferation had the effect of downregulating bcl-2 but upregulating p53 and p21 (Huang et al. 2010). Additionally, there is much evidence that trypsin causes mesenchymal cells to prolong their shape, inhibit respiration, and increase fermentation (Kellner et al. 1959). According to Huang et al. (2010), trypsin reduced the expression of proteins involved in regulating cell metabolism, growth, electron transport in mitochondria, and adhesion while increasing expression of those proteins which regulate apoptosis (Huang et al. 2010).

ASCs exhibit many remarkable properties but remain adult stem cells with limited self-renewal capabilities. Having no or very low telomerase activity (Bernardo et al. 2007; Baxter et al. 2004) causes these cells to lose about 50–200 nucleotides after every cell division (Zhao et al. 2009). The shortening of telomere length still occurs in vivo; however, under proliferative conditions, this rate is greatly increased. During the proliferation process, these cells gradually decrease their ability to proliferate and differentiate and become aged. This reduces their potential for application in medicine. Aging MSCs are arrested in cell division but still maintain metabolic activity. However, due to functional and molecular changes, the cells gradually increase in size, reach “fried egg” morphology, decrease the expression of specialized markers, and decrease their ability to differentiate (Wagner et al. 2010). Therefore, it is extremely important to determine what the appropriate steps are in ASC culture to maximize their efficacy in clinical use.

The aim of this study was to assess the characteristics of senescent ASCs in vitro after long-term expansion in order to provide insight into the extent of aging and limitations of multiple passages of culture. Understanding these limitations will benefit the application of expanded ASCs in research and treatment.

2 Materials and Methods

2.1 Isolation and Primary Culture of Adipose-Derived Stem Cells

Three samples of adipose tissues were collected from donors with consent forms. The study was

approved by an ethical committee. The adipose tissues were washed twice with PBS and then used to extract stromal vascular fraction (SVF) by Cell Extraction Kit (Regenmedlab, HCM city, Vietnam). Briefly, adipose tissues were mixed with super extract enzyme and incubated for 15 min. The adipose tissues were then centrifuged at 3500 rpm for 10 min to obtain SVFs. Next, the SVFs from the samples of adipose tissues were suspended in culture medium (MSCCult Basal Medium containing DMEM/F12 50/50, 1% Antibiotic-Antimycotic, and 10% FBS). The samples were plated in T-75 flasks for 7 days. Then, the medium was replenished with fresh medium.

2.2 ASC Expansion and Characterization

ASC samples from primary culture were subcultured with Trypsin/EDTA, 0.25% (Corning, NY, USA), and then cultured in MSCCult kit medium at 37 °C in a humidified atmosphere set at 5% CO_2. The cells were passaged when cell density reached approximately 10^3 cell/cm^2 (i.e., when they were in log phase) and before they reached full confluency (up to passage 15). Characterization of ASC senescence was performed on cells at passages 5, 10, and 15; cells were evaluated for their shape, immunophenotype, in vitro differentiation, tumor suppressor gene expression, beta-galactosidase expression, and mitochondrial activity.

2.3 ASC Shape and Dimension

Cellular shape and dimension of hASCs every five passages (from passage 5 onward) were evaluated under an inverted microscope (Carl Zeiss, Oberkochen, DE). The cells were also stained with Hoechst 33342 for further evaluation.

2.4 Immunophenotyping by Flow Cytometry (FCM)

ASCs at passages 5, 10, and 15 were subjected to immunophenotyping by flow cytometry (FCM) using the following monoclonal antibodies: CD14-FITC (fluorescein isothiocyanate), CD34 FITC, CD44-PE (phycoerythrin), CD45-FITC, CD73-FITC, Thy-1 (CD90)-FITC, Endoglin (CD105)-PE, CD166-PE, and HLA-DR-FITC, on a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). All antibodies were purchased from BD Biosciences except for CD73-FITC, Thy-1 (CD90)-FITC, and Endoglin (CD105)-PE, which were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX, USA).

2.5 Assessment of Trilineage Differentiation

Trilineage differentiation assay was conducted in 96-well plates (Corning, NY, USA) using the specific induced media above for adipocytes (adipocyte-induced medium): DMEM low glucose (Sigma-Aldrich, MO, USA), 89%; FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10%; antibiotic-mycotic (Sigma-Aldrich), 1%; dexamethasone (Sigma-Aldrich), 0.5 μM; 3-isobutyl-1-methylxathin-IBMX (Sigma-Aldrich), 0.5 μM; and indomethacin (Sigma-Aldrich), 50 μM. Moreover, chondroblast differentiation was induced by chondroblast-induced medium from StemPro™ Chondrogenesis Differentiation Kit (Thermo Fisher Scientific, Waltham, MA, USA). As well, osteoblast differentiation was induced by the use of osteoblast-inducing medium containing DMEM low glucose (Sigma-Aldrich), 89%; FBS (Gibco), 10%; Antibiotic-Antimycotic (Sigma-Aldrich), 1%; AsAP (apoptosis- and splicing-associated protein, Sigma-Aldrich), 50 μg/ml; dexamethasone (Sigma-Aldrich), 0.1 μM; and β-glycerophosphate (Sigma-Aldrich), 100 mM. After 7 days for adipocyte differentiation,

21 days for chondroblast differentiation, and 30 days for osteoblast differentiation, results were evaluated using an inverted microscope to visualize the bright red, orange-red color, deep red staining, which corresponded to alizarin red, safranin O, and oil red staining, respectively.

2.6 Evaluating Expression of Tumor Suppressor Genes

First, total RNA of hASCs at different passages was extracted using Easy-BLUE Total RNA Extraction Kit (iNtRON, Kyungki-Do, KR). Then, the extracted total RNA concentration was determined using a BioPhotometer Plus system (Eppendorf, Hamburg, DE). Finally, mRNA levels of p53, p16, and p27 were quantified with qRT-PCR technique on a LightCycler480II machine (Roche, Basel, CH) using qPCRBIO SyGreen 1-Step Lo-ROX Kit (PCR BIOSYSTEMS, UK). The reactions were established according to the manufacturer's instructions, with a total volume of 10 μl per reaction. Specifically, reverse transcription was set at 45 °C for 10 min, polymerase activation at 95 °C for 2 min, and cycling condition set at (95 °C for 5 s, 60 °C for 20 s) ×40 cycles. Melting curve analysis was done as recommended on the device. GAPDH was used as an internal control, and the relative quantitative results were determined.

2.7 Evaluating the Accumulation of Senescence-Associated Beta-Galactosidase

ASCs at different passages were reseeded in 96-well plates at the same concentration, at 10^4 cells per well overnight. After that, SA-β-GAL concentration was determined using Mammalian beta-Galactosidase Assay Kit (Thermo Fisher Scientific).

2.8 Evaluating the Activity of Mitochondria in the Aging of Human Adipose-Derived Stem Cells

Mitochondrial dysfunction was evaluated through FCM technique using MitoTracker™ Red CMXRos (Thermo Fisher Scientific).

2.9 Statistical Analysis of Data

Data from the assays above were processed by MS Excel 2016 and GraphPad Prism 7. Significant differences were defined if p-value < 0.05.

3 Results

3.1 ASCs Changed in Shape and Dimension

ASCs were cultured and continuously subcultured to passage 5, passage 10, and passage 15. At passage 5, they were relatively homogeneous; cells had the characteristic spindle-shaped, smooth cell surface. At passage 10, the cells maintained the characteristic MSC shape. However, some cells appeared to have structures like pseudopods, though longer and flatter. Unlike ASCs at passage 5, almost all ASCs at passage 15 exhibited the "fried egg" shape (Fig. 1). As well, their proliferation rate was significantly reduced. This observation was confirmed again in Fig. 2 when ASCs were stained with Hoechst 33342 dye. The cell shape gradually flattened and reached the "fried egg" morphology at passage 15.

In addition to the change in shape, the density of cells was also greatly reduced, as was the number of nuclei in the same field of view and the distance between one nucleus and another. At passage 5, the cells were very close together; at passage 10, the cells still adhered relatively close together, but the distance grew farther, while at passage 15, the cells were quite discrete, with very low cell density. The cell diameter also

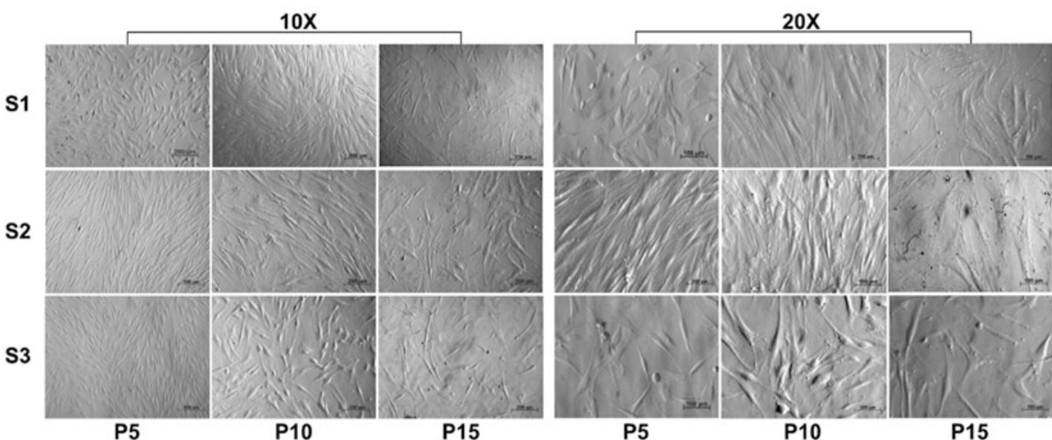

Fig. 1 Morphological change of ASCs in in vitro culture after subculture to passages 5, 10, and 15. ASCs were relatively homogeneous with spindle shape and smooth cell surface at passage 5, appeared some pseudopod structures at passage 10 and exhibited the "fried egg" morphology at passage 15. *ASC* human adipose-derived stem cell; *P5, P10, P15* passages 5, 10, and 15, respectively; *S1, S2, S3* samples ASC1, ASC2, and ASC3, respectively

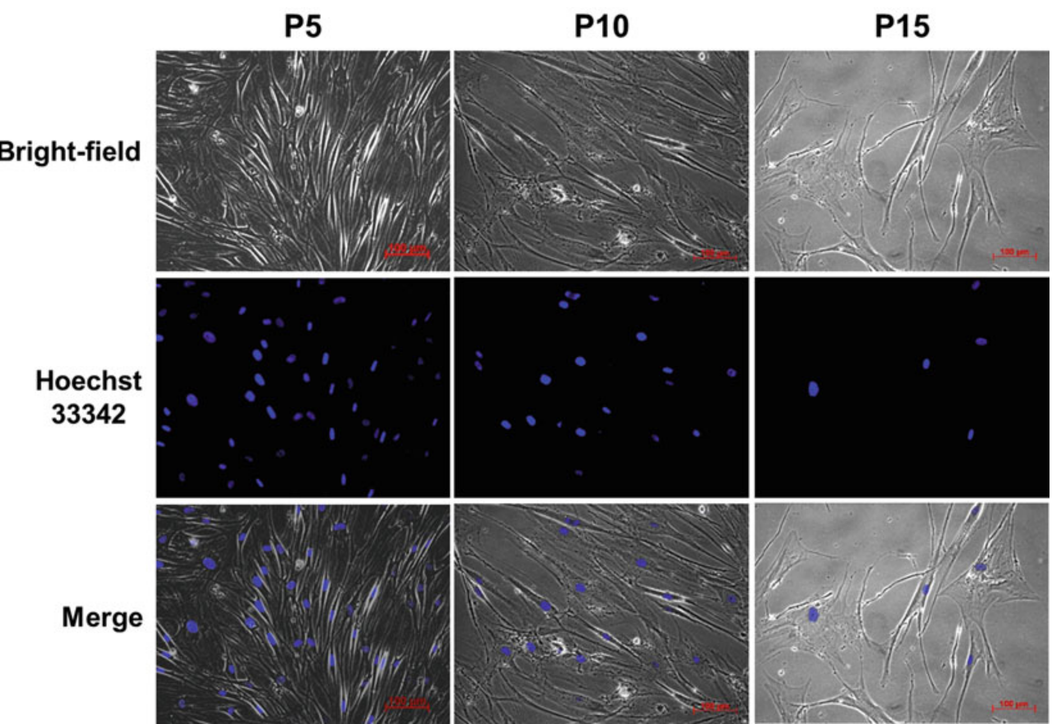

Fig. 2 The shape changing of ASCs in in vitro culture stained with Hoechst 33342 dye. As the passage grew progressively, the shape of the cell gradually grew and reached the shape of the fried egg at passage 15. At passage 5, the cells were very close together, less than at passage 10, and very far apart at passage 15. *ASC* human adipose-derived stem cell; *P5, P10, P15* passages 5, 10, and 15, respectively

changed after long-term culture. The results showed that cell diameter gradually increased when cell passage number increased. Indeed, at passage 5, the average cell diameter was relatively small, about 19.62 ± 1.84 μm (from 17 to 23 μm in diameter). At passage 10, the cell diameter significantly increased to 24.22 ± 4.84 μm, compared to the diameter at passage 5 ($p < 0.001$). By passage 15, cell diameter continued to increase to 25.72 ± 5.39 μm (Fig. 3).

The changes in cell diameter were also recorded by increasing the forward scatter (FSC) value in FCM analysis. The difference in size between passage 5 and passage 10 was greater than that between passages 10 and 15 (Fig. 4a). In addition to the increase in size, the side scatter (SSC) value increased accordingly. The SSC values in passages 10 and 15 were higher than in passage 5, and there was almost no difference between passages 10 and 15. In addition, the SSC plot in passages 10 and 15 had two distinct peaks, but there was only one peak in the plot for passage 5 (Fig. 4b).

3.2 Changes in Immunophenotyping

The results of the FCM analysis showed that ASCs did not express the standard hematopoietic markers (CD14, CD34, CD45, HLA-DR) at passages 5, 10, and 15. Indeed, the expression of CD14 at passages 5, 10, and 15 was 1.58%, 0.64%, and 1.65%, respectively. Meanwhile the expression of CD34 increased slightly but was negligible (0.17% at passage 5, 0.95% at passage 10, and 2.13% at passage 15). The expression of the CD45 at passages 5, 10, and 15 was 0.01%, 0.21%, and 0%, respectively. HLA-DR marker was not present in all cultures (Fig. 5).

In contrast, ASCs exhibited strong markers of MSCs, such as CD44, CD73, CD90, CD105, and CD166. Specifically, the expression of markers CD44 and CD73 were the highest (>95%) and remained constant across all three passages (5, 10, and 15). The expression of CD44 marker in passages 5, 10, and 15 was 97.09%, 97.10%, and 96.94%, respectively. The expression of CD73 marker over the subculture process was 98.53%, 98.27%, and 95.96% for passages 5, 10, and 15, respectively. Markers CD90 and CD166 showed mild relief but remained above 84.15% and 87.96%. More specifically, CD90 marker expression across passages 5, 10, and 15 was 95.66%, 91.12%, and 84.15%, respectively. CD166 marker expression was 98.97%, 98.09%, and 87.96%, respectively, for passages 5, 10, and 15. The CD105 marker showed a significantly reduced expression during in vitro culture; at passage 5, the expression of CD105 was very high, reaching 92.43%. However, the expression of this marker sharply decreased from passage 10 of culture (58.52%) and at passage 15 (48.87%) (Fig. 5). Thus, in our study, hASCs maintained the expression of MSC markers and did not express hematopoietic markers during culture.

3.3 Changes in Trilineage Differentiation

ASCs were transferred to 96-well plates with homogeneous density (10^4 cells/well) and induced to differentiate into osteoblasts, chondroblasts, and adipocytes. The results showed that despite several generations of subculturing (up to passage 15), ASCs retained their ability to differentiate into the three specific cell lines of MSCs. At passages 5 and 10, the substrates secreted during bone and cartilage differentiation were collected on the culture surface and stained with specialized dyes. At passage 15, cell density was less than the early passages which means the space between cells increased remarkably. In terms of adipocyte differentiation, there was no difference between the cultures (Fig. 6).

3.4 Expression of Tumor Suppressor Genes

Changes in expression of tumor suppressor genes p16, p27, and p53 were evaluated by assessing mRNA levels during in vitro ASC proliferation. In general, all three genes examined were reduced

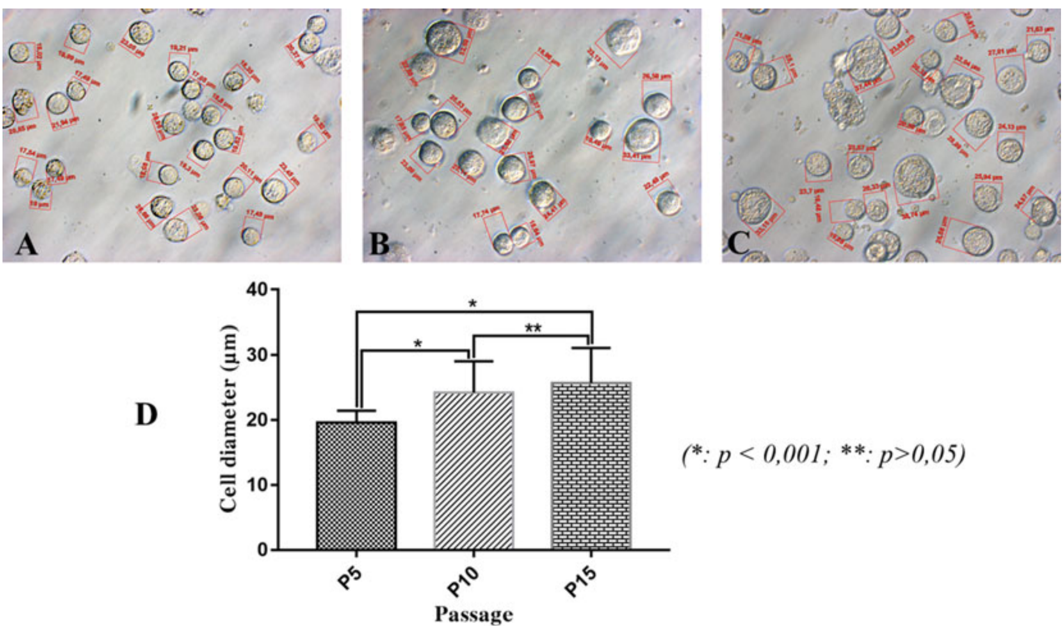

Fig. 3 Cellular diameter of ASCs increased from passages 5 to 15. The diameter of the ASCs was relatively small, with an average size of 19.62 ± 1.84 µm at passage 5, and increases to 24.22 ± 4.84 µm at passage 10 and to 25.72 ± 5.39 µm at passage 15. *ASCs* human adipose-derived stem cells; A, B, C photos taken in suspension of ASCs at passages 5, 10, and 15; *D* the average size of ASCs through subculture

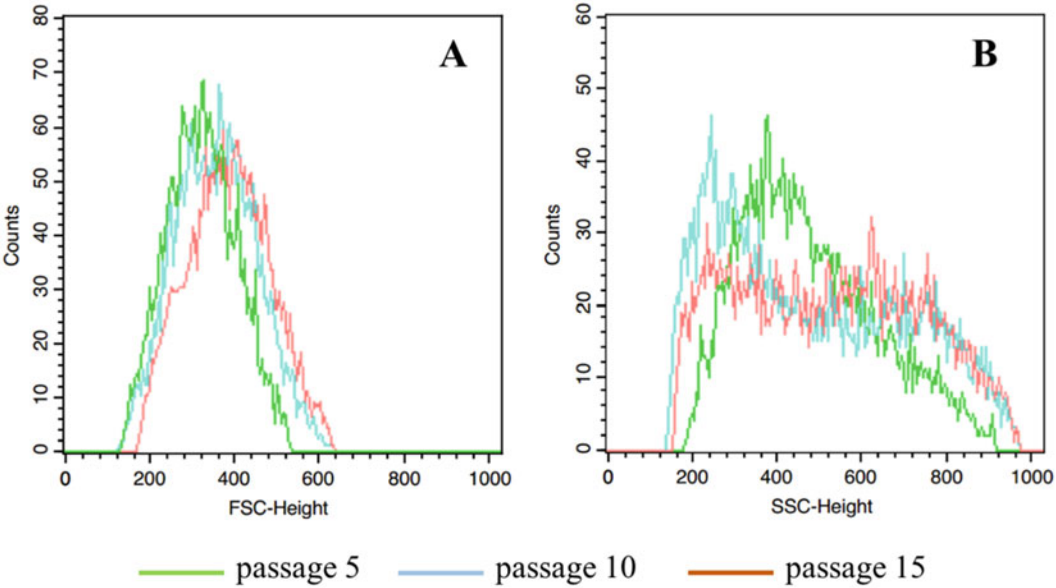

Fig. 4 Results of comparison between FSC (**a**) and SSC (**b**) values of ASCs between passages 5, 10, and 15. (**a**) FSC value increased through subculture, corresponding to the increase of cell size; **(b)** SSC value of passage 5 had a clear peak; after passage 5, SSC value increased and almost did not significantly change between passages 10 and 15; SSC values of passages 10 and 15 dispersed into two distinct peaks. *FSC* forward scatter, *SSC* side scatter, *ASCs* human adipose-derived stem cells

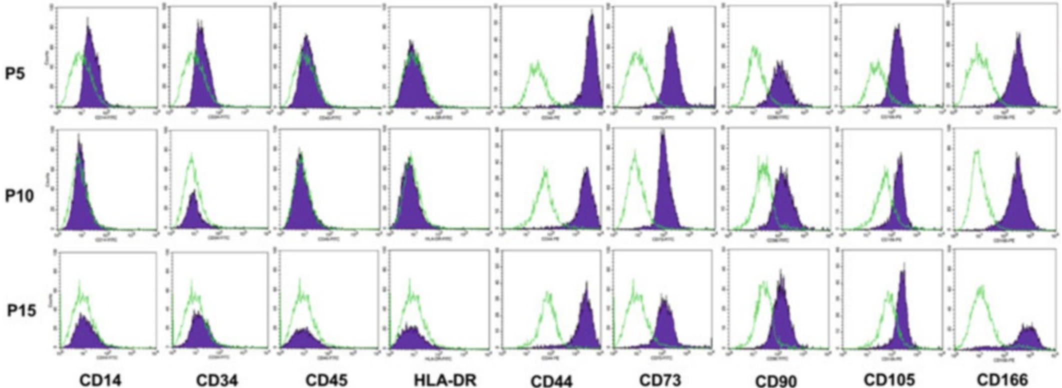

Fig. 5 Results of FCM analysis assessed the expression of hASC markers in in vitro culture. The expression levels at passages 5, 10, and 15 of CD14 were 1.58%, 0.64%, and 1.65%; CD34 were 0.17%, 0.95%, and 2.13%; CD45 were 0.09%, 0.21%, and 0%; CD44 were 97.09%; 97.10%, and 96.94%; CD73 were 98.53%, 98.27%, and 95.96%; CD90 were 95,66%, 91.12%, and 84.15%; CD105 were 92.43%, 58.52%, and 48.87%; CD166 were 98.97%, 98.09%, and 87.96%; HLA-DR was not expressed in all three passages of the culture; hASCs maintained their trilineage differentiation. *P5, P10, P15* passages 5, 10, and 15, respectively; *FCM* flow cytometry; *ASCs* human adipose-derived stem cells

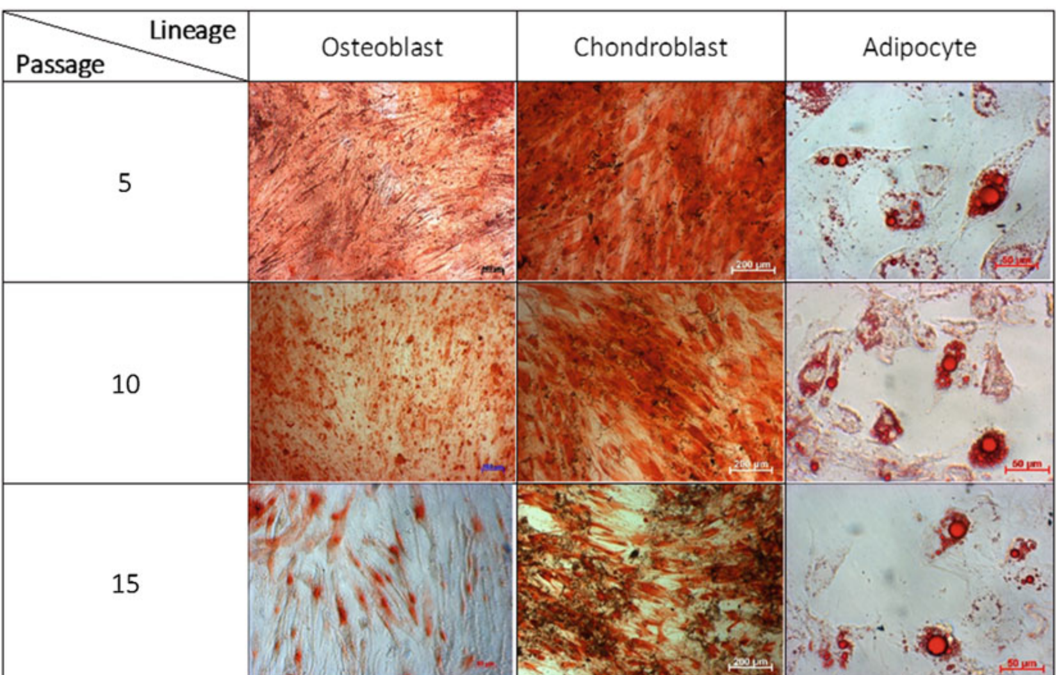

Fig. 6 Trilineage differentiation capacity of hASC in in vitro culture. Over 15 passages of subculture, ASCs have retained the ability to differentiate into bone, cartilage, and fat. There was a slight decrease in the ability to differentiate into bone and cartilage, and there was almost no difference in fat differentiation. *ASCs* human adipose-derived stem cells

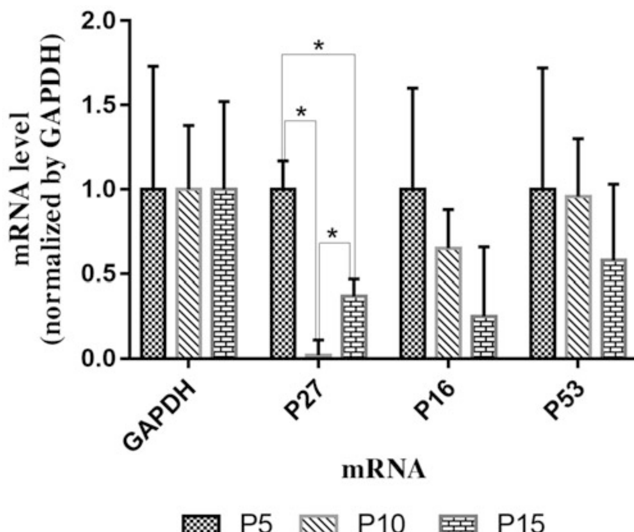

Fig. 7 Relative expression of p27, p16, and p53 of hASCs during in vitro culture. Genes (p27, p16, and p53) tended to decrease expression during culture. However, gene p27 exhibited the greatest reduction in expression ($p < 0.05$). The remaining genes, p16 and p53, showed no statistically significant decrease ($p > 0.05$). *: $p < 0,05$. *hASC* human adipose-derived stem cell; *mRNA* messenger RNA; *GAPDH* glyceraldehyde-3-phosphate-dehydrogenase; *P5, P10, P15* passages 5, 10, and 15, respectively

in culture. However, the decrease in the expression of genes p16 and p53 between passages of culture was not statistically significant ($p > 0.05$); this was due to the large variation between samples. The expression of p27 varied significantly during culture and was statistically significant ($p < 0.05$). Assuming that the expression of p27 in passage 5 of culture was 100%, the expression decreased sharply, to 2% in passage 10 and 37% in passage 15 (Fig. 7). Thus, tumor suppressor genes p16, p27, and p53 were all reduced during in vitro culture. Among them, gene p27 exhibited the most pronounced reduction; particularly, the sharp decrease occurred in the period from passage 5 to passage 10 but increased slightly during the period from passage 10 to passage 15.

3.5 Mitochondrial Dysfunction

The results showed that ASCs exhibited strong mitochondrial activity at passage 5 and significantly decreased from passage 5 to passage 10, corresponding to 96.17 ± 0.51% and 80.59 ± 2.35%, respectively. Subsequently, in the subsequent passages of culture, mitochondrial activity was almost unchanged and remained stable at 79.09 ± 1.75%. That was a reason that the rate of ASCs with lower mitochondrial activity increased significantly from 4.23 ± 0.56% in passage 5 to 19.68 ± 1.94% in passage 10 and remained stable until passage 15 (21.16 ± 1.70%) (Figs. 8 and 9).

3.6 Senescence-Associated Beta-Galactosidase Accumulation

In the culture process, SA-β-gal was virtually absent from passage 5 (0.004 ± 0.003). The amount of SA-β-gal in passage 10 increased by 3.75-fold compared to passage 5 of culture ($p < 0.001$), corresponding to 0.015 ± 0.003 absorption at 405 nm. The accumulation of this enzyme continued to increase slightly by 1.25-fold from passage 10 to passage 15 ($p < 0.05$), corresponding to 0.019 ± 0.003 absorption at the same wavelength (Fig. 10). However, in fact, when observing quantitatively, wells were stained with the substrate of the SA-β-gal enzyme in passages 5, 10, and 15; the color of these wells showed no significant differences.

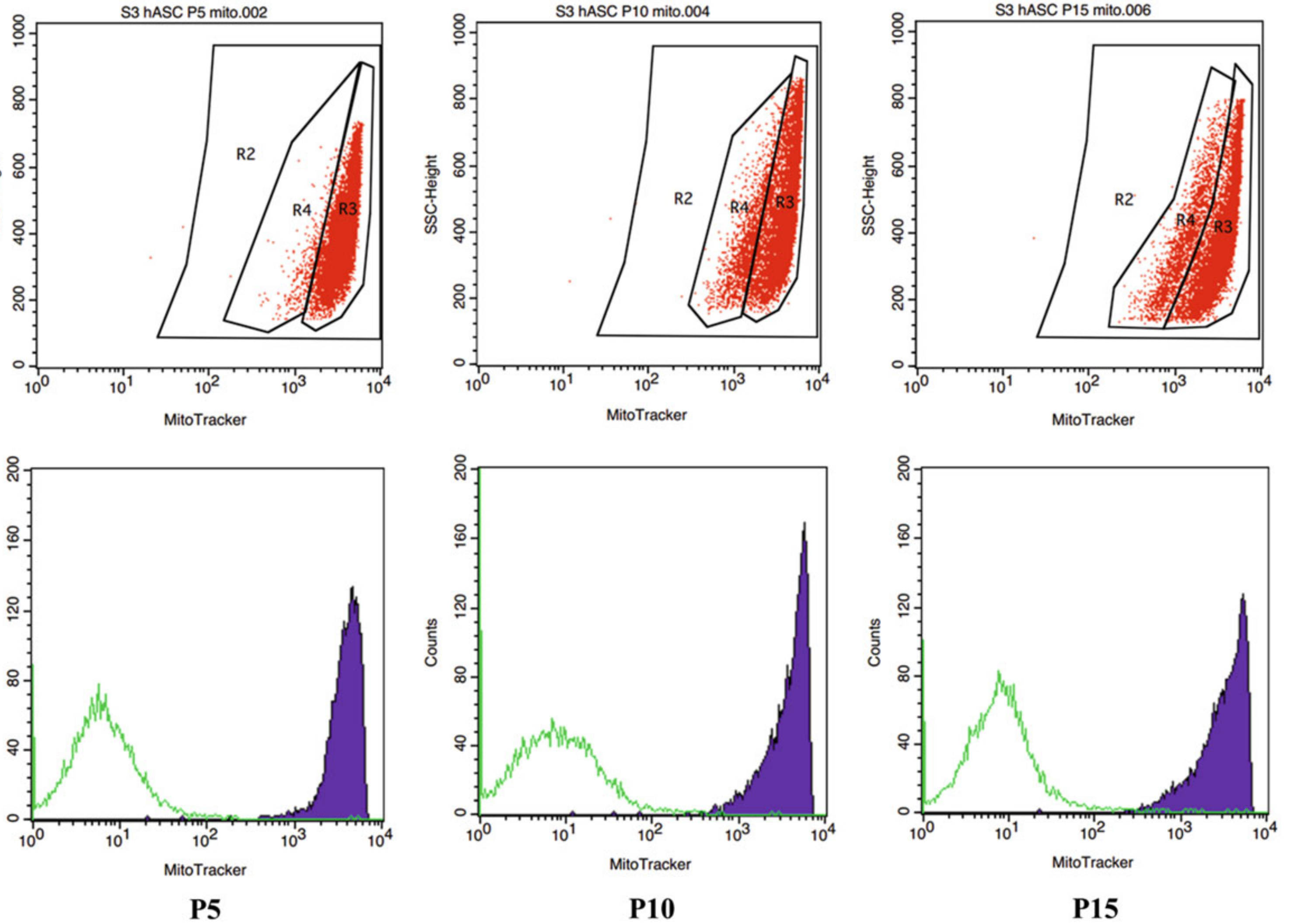

Fig. 8 Mitochondrial activity of hASCs in in vitro culture by FCM. The proportion of mitochondrial cells was greater than 98% in all passages 5, 10, and 15; however, the higher passage of culture, the higher the level of dispersion in the plot of mitochondrial activity. *P5, P10, P15* passages 5, 10, and 15, respectively; *R2* area with mitochondrial positive cell; *R3* region with strong mitochondrial positive rate; *R4* region with weak mitochondrial positive rate; *hASC* human adipose-derived stem cell; *FCM* flow cytometry

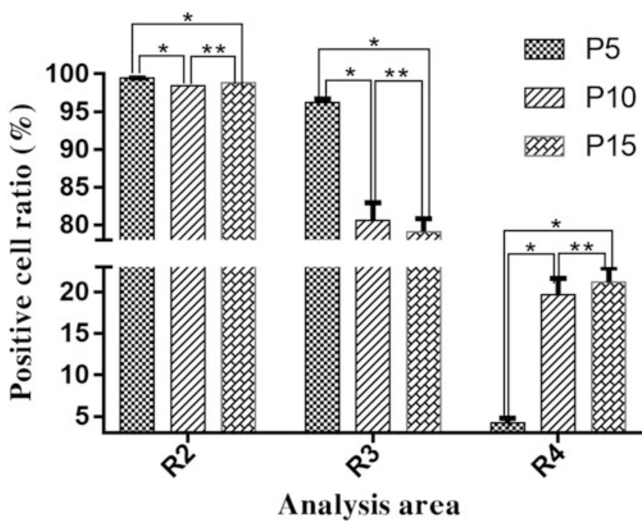

Fig. 9 Results of evaluation of mitochondrial activity of hASCs after long-term culture. For all passages, the rate of cells with mitochondrial activity was very high (R2 > 98%). However, there was a decrease in the rate of cells with strong mitochondrial activity (R3) from 96.17% in passage 5 of culture to 80.59% in passage 10 ($p < 0.001$); this ratio maintained stable until passage 15. *, p < 0,001; **, p > 0,05. *R2* area with mitochondrial positive cell; *R3* region with strong mitochondrial positive rate; *R4* region with weak mitochondrial positive rate; *FCM* flow cytometry; *P5, P10, P15* passages 5, 10, and 15, respectively; *hASC* human adipose-derived stem cell

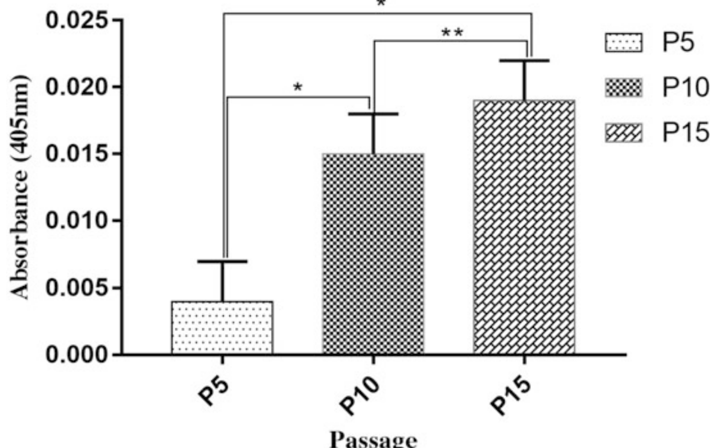

Fig. 10 The accumulation of SA-β-gal of hASCs in long-term in vitro culture. The SA-β-gal expression was very low in passage 5, up to 3.75 times in passage 10 ($p < 0.001$), and slightly increased, 1.27 times in passage 15 (when compared with passage 10, $p < 0.05$). *, p < 0,001; **, p < 0,05; *SA-β-gal* senescence-associated beta-galactosidase; *hASC* human adipose-derived stem cell; *P5, P10, P15* passages 5, 10, and 15, respectively

4 Discussion

In the body, the quantity of MSCs, in general, and ASCs, in particular, is not high. Thus, it is necessary to grow them in vitro to provide sufficient numbers of these cells for research studies as well as for treatment purposes in regenerative medicine (Strem et al. 2005; Lendeckel et al. 2004; Mosna et al. 2010). The number of patients treated with MSCs expanded in vitro has still been quite limited (Ikebe and Suzuki 2014). In

the culture process, MSCs may be altered, failing to adequately reflect their physiological and pathological roles compared to naïve cells (Pal and Das 2017). In particular, the problems of self-renewal and multi-lineage loss have taken place with high rates of MSC proliferation (Chosa and Ishisaki 2017). Moreover, there are potential risks of abnormalities in in vivo differentiation or neoplastic transformations, although evidence has only been found in mouse studies and usually after long periods of in vitro culture (4–5 months) (Mosna et al. 2010).

One of the main causes for this change is aging, which is a normal process in living cells, including stem cells. Indeed, ASCs are not spared from aging. The process of aging is more accelerated in in vitro culture, in spite of attempts to reproduce the physiological conditions as in the body. Therefore, the study herein aimed to characterize the aged ASCs during long-term expansion in culture.

Firstly, the shape and size of cells were recorded. At passage 5, the shape of the cells was relatively homogeneous and similar to fibroblasts, with smooth cell surface and an average diameter of 19.62 ± 1.84 μm. At passage 10, hASCs retained their shape; however, some cells appeared to have pseudopod structures, and cellular shape was flatter and longer (diameter increased to 24.22 ± 4.84 μm). At passage 15, almost all of the cells stopped growing and had a "fried egg" morphology. The average diameter increased to 25.72 ± 5.39 μm, and diameter reached greater than 38 μm. The increase in size of the culture was confirmed by an increase of the FSC value in the FCM analysis. This finding was consistent with reports by Wagner et al. (2010), which suggested that after the growth period outside the body, MSCs increased in size, flattened, and lost their ability to proliferate (Wagner et al. 2010; Chosa and Ishisaki 2017).

There were many theories that help clarify these changes during cell culture. For example, incubation with trypsin during cell separation could break down proteins outside the cell membrane. Meanwhile, it could be the link between the skeleton and the cell membrane (via extra-membrane proteins or transmembrane proteins) which promotes cellular morphological changes (Fletcher 2013). Therefore, the damage to proteins on the cell membrane could cause a change in cellular morphology. At the same time, many membrane proteins can act as receptors and are key mediators of the signaling pathways between cells or between the cell and the surrounding environment. Trypsin could damage the receptors on the surface of the cell and even cause cell death if exposed for a long time (Azari et al. 2010). In addition, it was shown that there was an increase in the SSC value from passage 5 to passage 10, but it was almost unchanged until passage 15. The main cause of the increase in granularity (or SSC value) of aging cells was presumably from an increase in the number of lysosomes containing lipofuscin, organelles, storage particles, and/or inclusions (Hwang et al. 2009). There have been several other studies which lend support for the increase of the complexity or granularity in growing cultures (Hwang et al. 2009; Estrada et al. 2013; Bakopoulou et al. 2017).

Secondly, changes in expression of certain markers were evaluated. Despite a long period of culture, ASCs were negative for hematopoietic markers CD14, CD34, CD45, and HLA-DR. In contrast, the expression of MSC markers, such as CD44, CD73, CD90, CD105, and CD166, was very high and consistent with many other studies (Dominici et al. 2006; Ong et al. 2014; Zhu et al. 2010; Mildmay-White and Khan 2017; Bertolo et al. 2017). In particular, expression of CD44 did not change during culture and was higher than 96% in all passages that were evaluated. Compared with passage 5, the expression of CD73 in hASCs at passage 15 decreased slightly but was still $\geq$ 96.94%. CD90 and CD166 expression decreased but remained high at $\geq$ 84.15% and $\geq$ 87.96%, respectively, at passages 10 and 15. The expression of CD105 was significantly reduced to 58.52% at passage 10 and 48.87% at passage 15. Thus, overall, the markers CD44, CD73, CD90, and CD166 are not specific for the aging of hASCs. CD105 can be a typical marker for assessing the aging of hASCs during in vitro culture. Our findings are consistent with

many other studies, such as that of Baer et al. (2010), Mangum et al. (2017), Ye et al. (2016), (Maredziak et al. 2016), and Athina Bakopoulou et al. (2017). In addition, there is strong evidence that CD105 is a useful marker for detecting MSCs in vivo (Lin et al. 2013). Thus, this marker can be used to assess aging of hASCs within the body.

Thirdly, functional assays or in vitro differentiation studies are necessary to demonstrate the ability of multi-lineage differentiation undergoing secondary culture (Mosna et al. 2010; Tamm et al. 2013). Muraglia et al. (2000) reported that MSCs cultured long time in vitro lost their self-renewal and multipotential capacity in a gradual fashion (Muraglia et al. 2000). In our study, at passage, hASCs retained the potential to differentiate into all three lineages: bone, cartilage, and fat. From that, we might initially conclude that the loss of differentiation was not characteristic of the aging process of ASCs, at least until passage 15. Previously, Baer et al. (2010) demonstrated that the fat differentiation ability of ASCs does not affect proliferation in vitro (Baer et al. 2010).

Normally, the aging process involves the accumulation of DNA damage and overexpression of cell cycle inhibitory proteins (tumor suppressor genes) (Lopez-Otin et al. 2013). Chromosome 4q21 contains the *INK4-ARF* locus encoding for three major tumor suppressor proteins, including P16INK4A, P19ARF, and P15INK4B (Matheu et al. 2009), which play important roles in inducing cellular senescence via P16INK4A/RB and P19ARF/P53 pathway (Sherr 2012). In our study, the expression of tumor suppressor genes p16, p53, and p27 all tended to decrease. It is worth noting that the dominant pathways in cellular senescence, notably P16INK4A/RB and P19ARF/P53, play the role of cross-regulating each other (Campisi 2013). Similarities in the expression results of these genes have been well established. According to Matheu et al. (2009), the increased expression of INK4/ARF genes reduced the aging marker as well as extended the life span of experimental mice (Matheu et al. 2009). On the contrary, Wagner et al. (2008) reported that genes on chromosome 4q21 increased during in vitro culture (Wagner et al. 2008; Signer and Morrison 2013).

However, the reason of this increase, whether due to cellular aging or induced by carcinogenic stimuli, remains unclear (Signer and Morrison 2013). Inferably, the expression of the tumor suppressor genes in our study might have decreased in correspondence to the reduction of oncogenic stress. In addition, the inactivation of P53 can cause MSCs to differentiate and lose their multipotent state, as reported by Boregowda et al. (2018) (Boregowda et al. 2018); thus, this might support our hypothesis. Furthermore, Gao et al. (2010) demonstrated that $p27^{+/+}$ MSCs tended to migrate to the tumor more than $p27^{-/-}$. Thus, reduced expression of p27 during culture may be beneficial at reducing the risks associated with transplantation (Li et al. 2016; Widder et al. 2016; Melzer et al. 2016).

Fifthly, ASCs can be characterized by their mitochondrial activity. Indeed, approximately 3 decades ago, it was known that the accumulation of mitochondrial mutations and abnormalities had important ramifications on aging and many degenerative diseases in humans (Linnane et al. 1989; Korolchuk et al. 2017). As compared to the DNA of nucleus, mitochondrial DNA is very sensitive to mutagenic agents (Lopez-Otin et al. 2013) but contains more than 1000 important genes (Wallace 2010). There is evidence that the number of mitochondria decreases in the course of aging (Bertolo et al. 2017; Bratic and Larsson 2013). In this study, we looked at the evaluation of mitochondrial activity as a criterion in evaluating the aging of hASCs during in vitro culture. Our results showed a reduction in the number of mitochondria in in vitro culture from passage 5 to passage 10 and then 15. This reduction is unavoidable in the culture of mature adult stem cells. However, our procedure limited the reduction of mitochondrial numbers in the process of aging while keeping the mitochondrial number stable in the last passages (from passage 10 to passage 15).

Sixthly, ASCs were evaluated for SA-β-gal expression. The expression of this enzyme can be detected from passage 5 onward, but the

expression is very low. In passage 10, the accumulation of SA-β-gal increased by 3.75 times compared with passage 5 ($p < 0.001$). From passages 10 to 15 of culture, the accumulation of SA-β-gal increased 1.27-fold ($p < 0.05$). This indicates that SA-β-gal is not strongly expressed in ASCs during culture. The results were similar to the results of Legzdina et al. (2016) and Wagner et al. (2009); SA-β-gal showed little or no expression in early passages but increased gradually during hASC culture (Wagner et al. 2009; Legzdina et al. 2016). Marędziak reported that ASCs in earlier passage exhibited lower SA-β-gal than those of late passages, although this difference was negligible (Maredziak et al. 2016).

The life span of MSCs, in general, varied considerably for a variety of reasons. These could include initial cell sources, culture conditions, cell proliferation modalities, etc. Muraglia et al. maintained the MSC population within 19–23 doubling times by use of complete media supplemented with fibroblast growth factor (FGF) (Muraglia et al. 2000). According to Wagner et al. (2008), MSCs that proliferated for 43–77 days (7–12 passages) show morphologic abnormalities, increased size, and reduced expression of specific markers and finally stop growing. In their study, fat differentiation potential was reduced, while bone differentiation potential was increased (Wagner et al. 2008).

If cells proliferate in vitro, it means the culture medium assumes the same role as in in vivo conditions. In other words, serum-supplemented medium is capable of supporting the survival and proliferation of cells by replacing hormones, nutrients, and buffer media (binding proteins and binding factors) in vivo (Barnes and Sato 1980). MSCs (in general) and ASCs (in particular) can be acquired and can proliferate in a variety of media, such as cytokine-free media, serum-rich media, serum-free media, and basic FGF (bFGF)-rich media (Mosna et al. 2010). In addition, FBS is usually added to culture media at concentrations of 10–20% to provide important proteins for cell adhesion and proliferation, as well as inhibit differentiation (Mosna et al. 2010). Efforts have been made to separate the key components in FBS that support the survival and proliferation of cells but have been largely unsuccessful (Barnes and Sato 1980). However, FBS can cause cellular toxicity when used at high concentrations and over a long period (Barnes and Sato 1980). In our medium, there was only 10% FBS, and thus, long-term culture of hASCs did not encounter this problem.

In addition, other components (such as phenol red, HEPES (N-2-HydroxyEthylPiperazine-N′-2-Ethane Sulfonic Acid), L-glutamine, etc.) which are added to the culture media can also cause certain effects. The presence of phenol red in the absence of serum may affect the internal sodium-potassium (Na-K) balance. However, this effect may be impaired by the addition of serum or pituitary hormones in the culture medium (Karmiol 2000). HEPES is quite toxic to some cell lines when used at high concentrations (25 mM) due to the formation of hydrogen peroxide (H_2O_2). This effect only occurs when media containing HEPES (along with riboflavin) are exposed for several hours under light (Zigler Jr. et al. 1985). Finally, L-glutamine is an unstable amino acid, which decays in the medium, producing ammonia, which may have negative effects on cells (Arora 2013).

In 2012, Nelson et al. introduced a concept of contagious aging, i.e., an aging cell can induce the aging of neighboring cells through cell-cell interactions (Nelson et al. 2012). Therefore, there may be a reciprocal interaction between aging and non-aging cells in the population. It is possible that in the early passages, the rate of aging cells remained small and the aging transmission was still low. By passage 10, there was more accumulation of aging cells; these cells almost ceased to proliferate in passage 15. This theory opens the possibility of prolonging the proliferation of ASCs, as well as other MSCs, by removing the aging cells from culture, thereby preventing the transmission of aging to other young cells.

Contrary to the negative effects on ASCs expanded in long-term culture, many factors derived from hASCs are resistant to the aging process, namely, decrease of SA-β-GAL activity

and accumulation of γH2AX (a phosphorylated isoform of the histone H2A at the sites of double-strand breaks). At the same time, these factors also reduced the production of inflammatory mediators along with oxidative stress products and reduced activation of mitogen-activated protein kinases (Tofino-Vian et al. 2017; Platas et al. 2016). Other studies have suggested that ASCs still have telomere activity, albeit weaker than in cancer (Jeon et al. 2011). According to Laura M. Pérez et al. (2015) and Schafer et al. (2016), the fat environment may reduce the characteristics of the adult stem cells (Perez et al. 2015; Schafer et al. 2016). Therefore, the separation of ASCs from the surrounding fat does not lead to all negative effects. In addition, Zhuang et al. (2015) showed that MSCs in the later passages have stronger immunosuppressive properties; indeed, they can be used for the treatment of host versus graft disease or other immunosuppressive disorders (Zhuang et al. 2015). Eventually, cellular aging is certain to occur in culture. Nevertheless, ways to slow down this process is an important matter that needs to be investigated further.

5 Conclusion

ASCs are the most popular MSCs in the human body. This study showed that ASCs can proliferate with minor changes in their phenotype, differentiation potential, or other characteristics, up to passage 10. Their shape, dimension, CD105 expression, beta-galactosidase activity, and mitochondria activity significantly changed at passage 15 compared to passage 5. These characteristics of senescence suggest that ASCs will proceed into the aging process after ten passages of culture, with changes in shape, beta-galactosidase levels, and mitochondria activity. In our study, hematopoietic markers and differentiation potential were nonsignificantly changed. The results of this study herein provide potential assays to evaluate ASC aging during ASC expansion for therapeutic applications.

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Authors' Contributions NCT, PVP, and KHTB designed the study and read and corrected the manuscript. NCT wrote the manuscript, proliferated hASC samples, and evaluated gene expression, mitochondrial activity, and beta-galactosidase accumulation. NCT, PVP, and KHTB evaluated mesenchymal characteristics. All authors read and approved the final manuscript.

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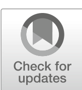

Sports Injuries: Diagnosis, Prevention, Stem Cell Therapy, and Medical Sport Strategy

Sadegh Rahim, Fakher Rahim, Kiarash Shirbandi, Behzad Bagheban Haghighi, and Babak Arjmand

Abstract

Sports injuries diagnosis, prevention, and treatment are the most important issues of sports medicine. Fortunately, sports injuries are often treated effectively, and people with damage recover and return to the sport in a satisfactory condition. Meanwhile, many sports injuries and complications can be prevented. In general, sports injuries include acute or chronic injuries. Given increasing in popularity, sports medicine doctors use stem cells to treat a wide variety of sports injuries, including damage to tendons, ligaments, muscles, and cartilage. Stem cell therapy to an injured area could be done through direct surgical application, stem-cell-bearing sutures, and injection. Stem cell therapy holds potential for repair and functional plasticity following sports injuries compared to traditional methods; however, the mechanism of stem cell therapy for sports injuries remains largely unknown. Medical imaging technologies provide the hope to ample the knowledge concerning basic stem cell biology in real time when transplanted into sport-induced damaged organs. Using stem cell treatment might restore continuity and regeneration and promote growth back the organ targets. Besides, using a noninvasive medical imaging method would have the long-time monitoring advantage to the stem cells transplanting individual. The multimodality imaging technique

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allows for studying acute pathological events following sports injuries; therefore, the use of imaging techniques in medicine permits the straight examination of dynamic regenerative events of specific stem cells following a sports injury in people.

Keywords
Medical imaging · Sports injuries · Stem cell therapy

Abbreviations

ADSCs	Adipose-derived stem cells
ASC	Adult stem cells
BM-MSCs	Bone marrow-mesenchymal stem cells
CBSC	Cord blood stem cell
CUP	Protector used in martial arts
ESC	Embryonic stem cell
MSCs	Mesenchymal stem cells
PBSCs	Peripheral blood stem cells
PRP	Platelet-rich plasma
SDF	Superficial digital flexor
T-MSCs	Tonsil-derived MSCs

1 Sport, Advantages, and Sports Injuries

1.1 Sport

Sport or exercise refers to activities or normal physical activities that are based on a range of universally agreed rules for recreational or quiz racing, personal joy, athleticism, skill, or a combination of these goals. There are various types of exercise, and humans devote an important part of their time, money, and interests not only as participants but also as spectators. Today's exercise in the world is transformed into a device that unites and connects people and interconnects fraternity and fraternity, and the main goal and task of the sport is to create friendship and friendship among sports people who, although in the field there are also competitions, are out of the squad as a friend of each other. Sport has many advantages, such as reducing anxiety and depression and increasing self-esteem. Participation in sports activities also helps to socialize, gain skills, and maintain healthy relationships with peers, and young people also argue that their exercise prevents alcohol and drug addiction (Daskalopoulou et al. 2017). In the course of intense exercise, the level of secretion of endorphins increases, and this is why athletes feel a sense of pleasure and relaxation after exercises (Maugars et al. 1995). Regarding the similarity between endorphins and opioid compounds, exercise can be a substitute for drug abuse in addicts (Wicklin 1994). As a consequence of exercise, blood flow increases in the brain, and this stream leads to oxygenation and better nutrition of brain neurons, which prevents stenosis of the arteries inside the brain (Dinoff et al. 2017). These effects of exercise prevent the forgetfulness and decline of mental variability in elderly. Exercise releases a kind of growth factor that can protect neurons from injury and damage and prevents individuals from Alzheimer's disease and Parkinson's disease (Ma et al. 2017). Moreover, physical activity decreases the risk of cardiovascular disease and stroke, as well as increases the amount of blood circulation in the body and prevents blood clots in the deep vein of the leg and strengthens the cardiovascular respiratory and the immune systems. The positive effects of exercise on the digestive and reproductive systems (Orio et al. 2013; de Oliveira and Burini 2009), as well as its cancer prevention effect should not be ignored (Anzuini et al. 2011). Nowadays interest in sports activities is increasing to need for the reduction of cardiovascular risks and increase in general health, as well as demand for fitness. Similarly, the rate of injuries to athletes has increased as a result of acute trauma and sports injuries.

1.2 Common Types of Sports Injuries

Sports injuries are injuries that typically occur when practicing at sports teams, competitions, training sessions, or fitness activities (Fig. 1).

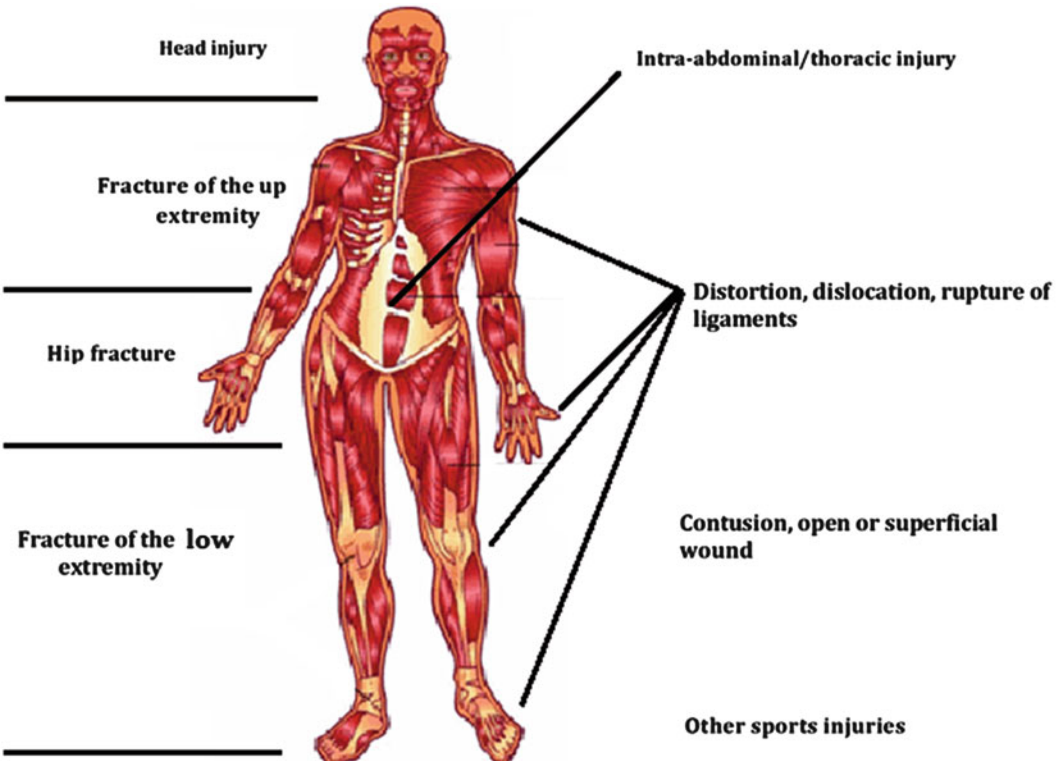

Fig. 1 The image represents the most common type and location of injury caused by exercise and sports activities on the human body

These kinds of injuries appear in a sport expert or trainer for a variety of reasons, such as unconventional training, inappropriate shoe, and nonspecialized equipment (Kreider et al. 2017). Sports injuries are divided into two general categories. The first group is acute traumatic injuries (Sandelin et al. 1988). These types of injuries are caused by the impact of opposing forces.

Acute traumatic injuries include fractures, a break, in a bone; bruising, contusion which is a skin-impaired rash and is a direct shock that causes swelling and bleeding in the muscles and other tissues of the body; stretch, any kind of tensile or tear of the muscles and tendons; scratches; and wound caused by tear, rupture of the skin that is usually deep and requires suturing (Assessment of Acute Traumatic Injuries 2016).

The second group of injuries is excessive or chronic injuries (Gronhaug and Norberg 2016). These types of injuries occur over time and are usually the result of repetitive exercises, such as running, tennis, and volleyball, and include fracture caused by pressure, fractures in the bone surface caused by excessive repetition of movements such as jumping basketball players, and tendon inflammation caused by constant stretching. Mostly, injuries caused by repetitive movements are less important than acute external injuries, but considering such injuries less important does not mean ignoring them.

1.3 Sports Injuries and Body Organs

Some people think that injuries only present in their hands, legs, and back during exercise and sport but should know that any organ in the body, such as the face, neck, head, sexual organs, hands, and feet, can be injured.

1.3.1 Head and Neck Injuries

Head injuries include stroke, bruising, fractures, and blood clots in the head. A stroke is caused by a shock or a sharp shaking on the head that causes the brain to stop functioning, or a shock to the brain. If the severity of the impact is too high or repeated, a stroke can cause serious damage to the brain. Fortunately, this complication is not common among adolescents. All injuries can be caused by trauma to the head area due to falling, severe shocks, head injury, and fracture or displacement of the neck.

Movement and fracture of the neck necks due to the injury usually caused by sudden head movement. The use of helmets in sports that are likely to have a high physical contact or when doing activities such as cycling and skating can always help to prevent head injuries. Neck injuries are often more dangerous. These types of injuries can be caused by sudden hits in sports such as climbing, skiing, horseback riding, gymnastics, driving, rugby, judo, or boxing.

1.3.2 Foot Injuries

Foot injuries include ligament elongation, cracking, heel pain, and inflammation. Because all body weight is on the legs and they are constantly put on a lot of pressure, they have favorable conditions for injury.

1.3.3 Genital System Injuries

If such injuries occur, men tend to be more likely to suffer than women. Men should always wear a protective cover and in some sports use a CUP (protector used in martial arts) to protect their genital organs from injuries.

1.3.4 Back Area Injuries

These injuries, which include torsion, cracking, and stiffness, appear due to the complexity or pressure of the back muscles due to bending or lifting. Such injuries often occur during sports such as football, hockey, weightlifting, gymnastics, and basketball.

1.3.5 Hand Injuries

Hand injuries include cracking, dislocation, and complexity and often occur in sports where physical encounters are high (such as football and hockey). Such injuries are due to the collapse of the hands and fingers and their flickering, severe pressure, or direct impact.

2 Stem Cell

2.1 Definition and Types

Stem cells are an undifferentiated and pluripotent cell line, which means the ability to transform and differentiate into a variety of cells and tissues, including neural tissue, muscle tissue, tissue, and bone tissue (Nae et al. 2013). Stem cells can be found in the production of cells and, ultimately, various tissues; therefore these cells of great importance in the prevention and treatment of various types of human diseases (de Lazaro et al. 2014; Ardeshiry Lajimi et al. 2013; Azizidoost et al. 2014, 2015, 2016; Dehghanifard et al. 2013; Ebrahimi et al. 2014; Ebrahimi and Rahim 2014; Rahim et al. 2013; Saeidi et al. 2014; Saki et al. 2013; Shahrabi et al. 2014). Due to the unique ability of stem cells, these cells today are attractive topics in biology and medical science. Also, research in this field will increase our knowledge of how the body grows from one single cell and, more importantly, to help understand the mechanism of replacing healthy cells with damaged cells. The stem cells are divided into three groups of embryonic stem cells, adult stem cells, and umbilical cord blood stem cells, according to their characteristics (Fig. 2).

The most important organs producing stem cells are bone marrow, umbilical cord, and placenta. Among these resources, the main advantage of the stem cells of the umbilical cord is that they are very basic and can be highly differentiated. Also, stem cells derived from bone marrow have a high degree of differentiation (Markoulaki et al. 2008). The two features of the stem cell include renewal and proliferation. Stem

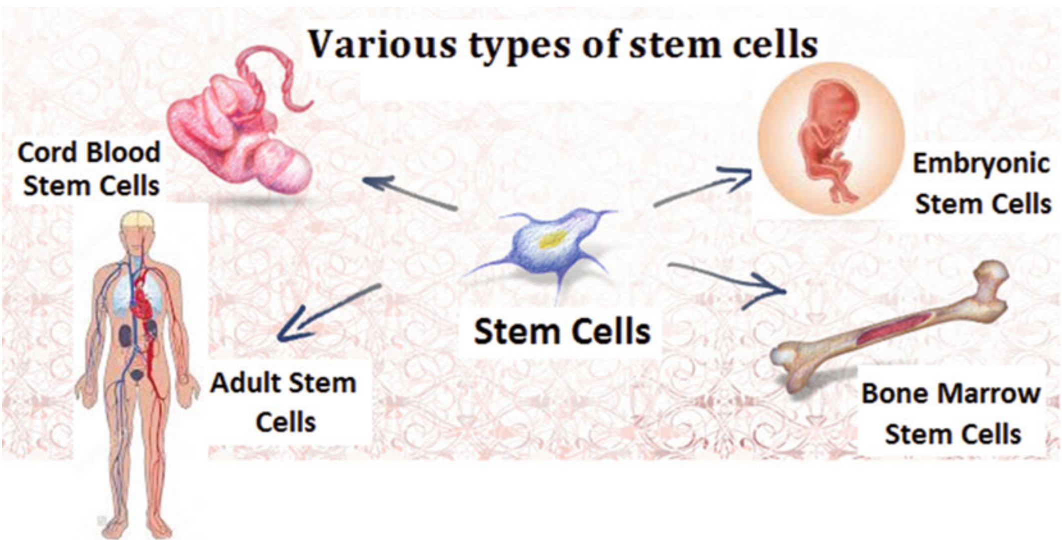

Fig. 2 Different types of commonly used stem cell therapeutic agents with their extraction source in human

cells are immortal cells that have the capability of their unlimited proliferation and remain undifferentiated and can also be differentiated to other stem cells and different cell types through mitosis (Altaner et al. 2013).

2.2 Embryonic Stem Cell (ESC)

These stem cells existed in the body of the embryo during the first weeks of its formation, which means the cells that make up the body of the human embryo (Shroff et al. 2017). It is clear that these cells can form different types of tissues and organs. These cells are taken from the internal cell mass of the 16–14-day-old fetus and are able to make all the cells and tissues of a person.

2.3 Cord Blood Stem Cell (CBSC)

These cells are similar to the embryonic stem cells that present in the blood of the umbilical veins. At the birth of the baby, these cells can be removed by cutting the umbilical cord from the blood of umbilical veins. These cells are less capable of converting into tissues and organs than embryonic stem cells, but their differentiation is much easier. The cord matrix called Wharton's jelly is the source of adult mesenchymal stem cells.

2.4 Adult Stem Cells (ASC)

A type of stem cells that separate from the tissues of a mature adult after birth is called adult stem cells. Hematopoietic stem cells in the bone marrow, brain, liver, and other tissues are of this category, which have the ability to differentiate into some tissues. Although the names of these cells mean the cells of the adult, these cells are present in the body from birth to puberty. So far, it has been found that stem cells from the beginning of the embryo's development to the end of life also refer to adult stem cells based on the ability to differentiate into tissues and organs.

3 Clinical Use of Stem Cells for Various Types of Sports Injury

So far, closed to 30 studies used various types of stem cells in the treatment of different types of sports injuries. Athletes, whether they are still in exercise or retired, have a strong need for ability

to recover using stem cell regeneration, due to severe injuries such as bone fractures, ligament dilatations, and cartilage wear, which have been seen during exercise. If the rehabilitation ability of these stem cells is proven, these cells can be the latest achievement in sports medicine.

3.1 Muscle Healing

So far we found only five studies that reported using stem cell therapy for post-sports injury muscle repair (Table 1). Bone marrow-mesenchymal stem cells (BM-MSC) are the primary interface for muscle repair and reconstruction. These multi-species cells regenerate by migrating to the target area and differentiating into muscle cells. However, its effect as a stem cell is minimal, and its use in the treatment of sports injuries is considered to be ineffective (Ferrari et al. 1998). Some studies reported the effective and well-defined role of the non-myogenic, non-satellite stem cell fraction is the remodeling, tissue maintenance, and initiation of beneficial adaptations post-eccentric exercise (Boppart et al. 2013). On the other hand, human adipose tissue-derived BM-MSC was reported to be more effective in producing muscle from bone marrow-mesenchymal stem cells and synovial membrane (de la Garza-Rodea et al. 2012).

Muscle repair is done by adults in BM-MSC, which has the ability to occupy the niche of the stem cells in the muscle after injury. In addition, after sports injury, muscle fibers that have been involved in the regeneration by BM-MSCs are reported much more than anywhere else. This indicates that BM-MSCs can be considered as a source of muscle cell repair (LaBarge and Blau 2002). Bruno et al. reported that BM-MSC treatment increased muscle fiber and led to the presence of mature muscle fibers after 28 days of muscle damage. However, there was no

Table 1 Studies that reported using stem cell therapy for post-sports injury muscle healing

Study ID	Study type	Follow-up	Study sample	Intervention	Outcome	Country
Ferrari, 1998	Ex	–	C57BL/six transgenic mice	Bone marrow-derived myogenic progenitors	These cells could possibly be used to target genes with therapeutic goals and considered as an alternate approach for treatment of muscular dystrophies	Italy
LaBarge, 2002	Ex	6 months	C57BL/six transgenic mice	Bone marrow-derived cells (BMDC)	Could result in an involvement to as many as muscle fibers environmental	USA
de la Garza-Rodea, 2012	Ex	–	Human (three patients)	MSCs	To be the best choice for the treatments of myopathies, due to their proficient involvement to myo-regeneration, great ex vivo expansion potential, and their harvesting	Canada
Hwang, 2013	Ex	4 weeks	BALB/c nude mice	Human adipose-derived stem cells (h-ADSCs)/basic fibroblast growth factor (bFGF)	The combination therapy caused revascularization, functional recovery, and reinnervation proposing this approach as a promising treatment for skeletal muscle regeneration	South Korea
Bruno M. Andrade, 2015	Ex	28 days	Wistar rats	BMMC	Speed up and advance function recovery of muscle	Brazil

EXP experimental

difference in the deposition of collagen in the two groups. Study of the markers of skeletal and smooth muscle cells revealed the apparent integration of BM-MSC in the muscle. These findings suggest that stem cell transplantation accelerates and increases muscular function in re-injury models (Andrade et al. 2015). Another study showed that the combination of adipose-derived stem cells (ADSCs) could lead to a complete remission of skeletal muscle in muscle rupture. As a result, ADSCs treatment showed a positive effect on functional, vascular, and neuromuscular regeneration for muscle reconstruction as well as suggests stem cell therapy as might be a promising treatment for sports injury (Lee et al. 2014; Hwang et al. 2013).

3.2 Tendon Healing

The details of available studies have been given in Table 2. In regarding tendon repair using stem cell therapy, the effect of BM-MSC fibrin gel on Achilles tendon of rabbits has been compared with the simple fibrin gel and reported that following 3-week follow-up, the modulus of elasticity was 32% greater and I-type collagen fibers percentage improved. Nevertheless, there was no significant change between study groups at 6 and 12 weeks (Chong et al. 2007). In another study, Achilles tendon injuries in rabbits include two PLGA scaffolds cultured with BM-MSCs or a simple scaffold compared to the group without any harm. The formation of new tissue and reconstruction in the BM-MSCs group was higher. In

Table 2 Studies that reported using stem cell therapy for post-sports injury tendon healing

Study ID	Study type	Follow-up	Participant	Intervention	Outcome	Country
Ouyang, 2003	Ex	12 weeks	Rabbit Achilles tendon	BMSC	This approach has the potential to restore structure and function, as well as regenerate and repair gap defect of Achilles tendon	Singapore
Chong, 2007	Ex	12 weeks	Rabbit Achilles tendon	BMSC	Can improve histological and biomechanical factors in the early tendon healing	Singapore
Godwin, 2011	Ex	3 years	113 horses	MSC	Is a relatively safe approach and appears to decrease the reinjure rate	UK
Komatsu, 2016	EX	4 weeks	36 male nude rats/3 male SD rats/10 GFP positive transgenic male rats	Tendon stem/ progenitor cell (TSC)	This procedure significantly improved histological condition and collagen content at both 2- and 4-week post-implantation May efficiently improves tendon remodeling in the early tendon healing	Japan
Kraus, 2016	Ex	12 weeks	60 male Lewis rats	MSC-LV-eGFP/ MSC-LV-bFGF	Did not show positive impacts on tendon remodeling in a long-term follow-up. Remarkably, stem cells had barely any impacts on biomechanical parameters in later stages	Germany
Lee, 2017	Ex	4 weeks	57 Sprague Dawley rats	MSC	Biomechanically improved tendon healing and suggest that these cells may be able to differentiate into the tenogenic lineage and contribute to tendon healing as well	South Korea

Ex Experimental

the BM-MSCs group, modulus of elasticity was 62.6% and tensile strength 87% of the original tendon; thus it seems that BM-MSCs have a long-lasting effect. The results suggest that the allogeneic BM-MSCs have the potential to regenerate and repair gap defect of Achilles tendon and to efficiently repair structure and function in the injured tissue (Ouyang et al. 2003). There is evidence that MSCs can help in the tendon recovery. MSCs cultured in fibrin glue in half of the mice were performed; the results of this experiment emphasized the increase of resistance to breakdown and pressure (Gulotta et al. 2012). Studying 19-superficial digital flexor (SDF) of tendon inflammation showed that the use of ADSCs and platelet-rich plasma (PRP) is a low-risk solution for treating tendon inflammation in the animal models. By studying the effect of pressure on the SDF tendon and its improvement by MSCs, it was reported that MSCs have long-term efficacy, and this treatment can also be used for human specimens (Godwin et al. 2012). Recently, a systematic review not advised the stem cell therapy for tendon disorders in clinical practice is currently (Pas et al. 2017).

3.3 Ligament Healing

Ligament is disposed to degeneration and injury and has poor healing potential, and, with presently unsuccessful management strategies, stem cell therapies may afford a favorable novel treatment choice (Table 3). A survey of 43 randomly selected patients concluded that BM-MSCs are limited in adult ACL regeneration (Silva et al. 2014). An animal study of the effect of stromal mesenchymal cells was concluded on ACL restoration on 18 rats and showed that these cells can be used to some extent to improve laceration in the ACL ligament (Kanaya et al. 2007). Figueroa et al. concluded a study that the use of these mesenchymal stem cells (MSCs) along with collagen type 1 scaffolds to enhance the recovery of ACL and to provide an attractive tool in the treatment of sports injury-induced ACL ruptures (Figueroa et al. 2014). A study that was performed on proximal suspensory ligaments rapture in an hours model showed that allogeneic tenogenically induced MSCs play a positive role in the treatment of these lesions (Vandenberghe et al. 2015). Chamberlain et al. conducted a study to treat ligament and tendon injuries with MSCs in order to modulate their inflammatory response and reported unconditioned MSCs to improve ligament healing by stimulating a stronger, paracrine-mediated immunosuppressive response (Chamberlain et al. 2017). Recent review revealed that the ligament-derived stem cell (LDSC), as well as LDSC niche as a vital component for the survival and function of stem cell participating in various physiological systems, could be considered as the therapeutic potential of ligament degeneration and injury (Lee et al. 2017). Tendon-bone healing (TBH) following ACL reconstruction is a complex procedure, which considerably impacts on patients' prognosis. TBH naturally results in fibrous scar tissue; thus, approaches to promote TBH are essential for quick and satisfactory functional recovery. Recently, a review described the current understanding of TBH and summarizes the current available stem cell therapy and showed that stem cell therapy may help to improve the healing process and gives a reliable fibrocartilage tendon-bone interface (Hao et al. 2016).

3.4 Bone Healing

Bone fracture healing using promising and new therapeutic processes may be particularly adequate for the treatment of athletes to permit a safe and stable return to sports (Table 4). Vannini et al. published results for 140 athletes treated with MSC. In this study, which focused on talus lesions in athletes, the results of the return of athletes undergoing treatment to satisfactory sports were significant and interesting (Vannini et al. 2017). Weel et al. developed a stem cell therapy protocol in a clinical trial study for the treatment of metastatic bone fractures (Weel et al. 2015). They provide evidence on the effectiveness of concentrated blood and bone marrow aspirate (cB + cBMA) in the treatment of bone fractures. Conceded bone-regenerating ability

Table 3 Studies that reported using stem cell therapy for post-sports injury ligament healing

Study ID	Study type	Follow-up	Participant	Intervention	Outcome	Country
Kanaya et al. 2007	Ex	4 weeks	Ninety-eight 12-week-old male Sprague-Dawley rats anterior cruciate ligament (ACL)	Mesenchymal stem cells (MSCs)	This technique was efficient for the healing of partially torn ACLs, gave insights into the effects on ligament injuries, and their forthcoming therapeutic uses	Japan
Silva et al., 2012	Ex	–	Anterior cruciate ligament (ACLs)	Mesenchymal stem cells (MSCs)	Do not seem to accelerate graft-to-bone healing in ACL rebuilding	Portugal
Figueroa et al., 2013	Ex	12 weeks	Ten New Zealand rabbits anterior cruciate ligament (ACL)	Mesenchymal stem cells (MSCs)	Causes an enhancement of ligament regeneration and is a potentially interesting technique for improving the treatment of ACL ruptures	Chile
Vandenberghe et al., 2015	Ex	12 weeks	Endinopathies	Mesenchymal stem cells (MSCs)	Cause a positive evolution of proximal suspensory ligament desmitis	Belgium
Chamberlain et al. 2017	Ex	–	Macrophages	Mesenchymal stem/stromal cells (MSCs)	Improve ligament and tendon healing	USA

Ex Experimental

Table 4 Studies that reported using stem cell therapy for post-sports injury bone fracture healing

Study ID	Study type	Follow-up	Participant	Intervention	Outcome	Country
Taiani et al. 2014	EX	4 weeks	Mice	Murine embryonic stem (ES)	Provide a novel insight into using stem cell therapies for bone injuries	Canada
Weel et al. 2015	Randomized controlled trial (RCT)	12–18 weeks	50 patients	Bone marrow	Effective treatment of MT-V stress fractures	Netherlands
Vannini et al. 2016	Randomized controlled trial (RCT)	48 months	140 patients (mean age 30.3 ± 10.5 years) with focal OLT	Bone marrow-derived cell transplantation (BMDCT)	Good clinical findings that were timely robust in athletes, permitting a return to sports	Italy

subsequent to a long bone fracture without surgical intervention application of BM-MSCs is a feasible, minimally invasive therapeutic option; thus, given clinical successes along with recent advances in applying and biology of MSCs, it leads us to a significant opportunity to develop a potential, novel, and safe therapeutic MSC-based approach to critically clinical strategies for bone regeneration (Watson et al. 2014). Taiani et al. conducted a study on an estrogen-deficient mouse model to test the efficacy of a murine embryonic stem (ES) on impaired healing fracture and introduced a novel insight into the use of stem cell therapies for bone injuries (Taiani et al. 2014). A broad variety of factors are known to induce the healing in bone fracture conditions; however, the influence of MSCs on bone fracture healing has not been fully studied. Zhang et al.

developed a mathematical model showing the change in biological processes within the fracture callus, which includes special features such as reduction in MSCs number locking for a system that acts on healing in this context (Zhang et al. 2017). Their findings could potentially lead to patient-specific solutions and thus achieve optimal healing outcomes in bone fracture conditions.

3.5 Meniscus Healing

A meniscus is a type of cartilage that exists in the knee joint and includes two types; each rests between the femur and tibia. It is made of tough cartilage and conforms to the surfaces of the bones upon which they rest. A meniscus tear is often caused by twisting or turning quickly, often with the foot planted, while the knee is bent. There is a huge amount of preclinical researches on the use of different types of stem cells in different experimental settings, which reported good results in terms of tissue formation for regenerate meniscus in tearing injury (Moradi et al. 2017). Many studies reported controversial results regarding the use of stem cells in the meniscus tearing injury healing process (Table 5). Moradi et al. conducted a study using adipose-derived mesenchymal stem cell (ADSC) to regenerate meniscus in tearing injury and study its contribution in the healing process and suggested that ASC has no significant involvement in the meniscus tearing injury healing process (Moradi et al. 2017). Hatsushika et al. stated that intravenous injection of allogeneic MSCs leads to increased meniscal restoration and protection in the middle part of the articular cartilage of the femur in the pig model (Hatsushika et al. 2014). Synovial-derived MSC is a possible therapy for meniscus regeneration. In this context, Katagiri et al. investigated whether using aggregates of synovial-derived MSCs regenerated meniscus more effectively in an animal model and showed that these cells can regenerate meniscus more effectively and enhance the healing process (Katagiri et al. 2013). Moreover, Nakagawa et al. investigated whether synovial-derived MSCs promote healing after meniscal repair with induction of cells to an extended longitudinal tear of avascular area in an animal model and indicated that stem cell therapy improves and promotes healing process (Nakagawa et al. 2015). Whitehouse et al. conducted a mixed experimental and clinical trial study using undifferentiated autologous MSCs to integrate meniscal tissues and reported that these stem cells may provide a safe way to augment avascular meniscal repair in individuals suffering sports injury (Whitehouse et al. 2017). Many studies have broadly characterized and confirmed that tonsil-derived MSCs (T-MSCs) are safe potential cell sources for bone (Moon et al. 2016; Jeong et al. 2016; Kim et al. 2017) and cartilage (Park et al. 2014; Park et al. 2015) healing applications. Koh et al. examined the effects of T-MSCs for meniscus tissue regeneration and highlighted a novel cell-based strategy combined with biomaterials designs for meniscus regeneration (Koh et al. 2017). Kremer et al. performed a study to investigate using 3D co-culture constructs of primary equine MSC for meniscal regeneration and revealed encouraging findings for further development of this scaffold-cell combination for meniscus tissue engineering (Kremer et al. 2017).

3.6 Cartilage Healing

Cartilage with a little capacity to repair itself owing to the lack of a blood supply, nerves, or lymphangion is a rubber-like padding, resilient, and smooth elastic tissue, which covers and protects the ends of long bones and many other body components. Various efforts have been done so far to shade a light on the use of different stem cells for cartilage healing (Table 6). Epidemiologic evidences on the management of cartilage defects present many clinical challenges to translate basic science to the clinical practice that revealed a lack of connectivity between the preclinical and clinical data, as well as a hugely heterogeneous nature of using MSCs (Goldberg et al. 2017). Other emerging evidences about the

Table 5 Studies that reported using stem cell therapy for post-sports injury meniscus healing

Study ID	Study type	Follow-up	Participant	Intervention	Outcome	Country
Katagiri et al. 2013	Ex	3 weeks	Rats	Mesenchymal stem cells (MSCs)	More effectively than intra-articular injection of synovial stem cells	Japan
Hatsushika et al. 2014	Ex	4 weeks	Pigs	Synovial mesenchymal stem cells (MSCs)	Intra-articular injections seem to improve meniscus regeneration and provide protection	Japan
Nakagawa et al. 2015	Ex	12 weeks	Pigs	Synovial mesenchymal stem cells (MSCs)	Promoted healing after meniscal repair in animal model	Japan
Moradi et al. 2017	Ex	7 months	New Zealand rabbits	Adipose-derived mesenchymal stem cell (ASC)	Can successfully regenerate meniscus in tearing injury	Iran
Whitehouse et al. 2017	EX prospective, open-label first-in-human safety study	13 weeks 2 years	Sheep Human	Undifferentiated autologous mesenchymal stem cells (MSCs)	A harmless way to augment avascular meniscal repair	UK
Koh et al. 2017	Ex	16 weeks	New Zealand rabbits	Tonsil-derived T-MSCs	Propose a novel strategy pooled with biomaterials designs for meniscus regeneration	Korea
Kremer et al. 2017	Ex	12 weeks	Horses	Primary equine MSCs	The findings inspire further progress of this scaffold-cell combination for tissue engineering of meniscus	Germany

use of stem cells in the field of cartilage regenerative medicine showed growing systematic understanding of the application of stem cells and several advanced strategies for cartilage healing (Wang et al. 2017). Moreover, other systematic reviews on using adipose, bone marrow, and synovial-derived MSCs for cartilage repair suggested that considering patients present with diverse problems, there is a need for an array of solutions that need to be cautiously aligned to the individual's necessities in the field of cartilage healing (Fellows et al. 2016).

Nakamura et al. concluded a preclinical study using MSCs in the pig model and concluded that there is better result in repairing the cartilage over 12 weeks (Nakamura et al. 2013). Dashtdar et al. performed a study of the effect of chitosan PVA composite hydrogel against alginate as a carrier of potential MSCs for the treatment of focal cartilage defects and suggested that these cells can help the treatment of injured cartilage slowly (Dashtdar et al. 2015). Saw et al. in a clinical trial treated 55 people with grade 3 and 4 cartilage rupture with peripheral blood stem cells (PBSCs) and concluded that these cells resulted in an improvement of the quality of articular cartilage repair (Saw et al. 2013). Nejadnik et al. compared the clinical efficacy of first-generation autologous chondrocyte implantation with autologous BMSCs in patients who underwent cartilage repairs and reported that these cells could be considered as an effective therapy in repairing articular cartilage (Nejadnik et al. 2010). It causes less knee surgery, reduces costs, and minimizes complications. Gobbi et al. in a cohort study of 50 patients concluded that cartilage repair by MSCs had successful medium-term outcomes independent of age or lesion size (Gobbi and Whyte 2016).

Table 6 Studies that reported using stem cell therapy for post-sports injury cartilage healing

Study ID	Study type	Follow-up	Participant	Intervention	Outcome	Country
Nejadnik 2010	Observational cohort	24 months		Bone marrow-derived mesenchymal stem cells (BMSCs)	Using this approach leads to less required surgery, less costs, and minimized donor-site morbidity	
Nakamura T 2013	Ex	12 weeks	Articular cartilage	Mesenchymal stem cells (MSCs)	Better treatment outcomes in cartilage regeneration associated with autologous synovial stem cell	Japan
Dashtdar 2013	Ex	3 weeks	Focal cartilage	Mesenchymal stem cells (MSCs)	Provides comparable treatment outcomes to that of formerly established ones, showing the potential use of this stem-cell-based combined hydrogel for clinical use in cartilage repair	Malaysia
Saw et al. 2013	A randomized controlled trial	6 months	Fifty patients aged 18–50 years with international cartilage repair society (ICRS)	PBSC	Resulted in an improvement of the quality of articular cartilage repair	USA
Jiang et al. 2016	–	–	Articular cartilage	Mesenchymal stem cells (MSCs)	These insights increase our biological knowledge of cartilage and may improve the success of chondrocyte-based treatments	China
Gobbi et al. 2016	Cohort study	5 years	Fifty physically active patients	HA-BMAC	Provides superior treatment outcomes and more durable cartilage repair at midterm follow-up	Italy

4 Conclusion

The new era of professional sports relied on sports medicine more than ever. Despite the fact of its low profile compared to the other medical specialty, it is accelerating rapidly with the advent of improving the performance and health of athletes. Innovations in this field have created new strategies for managing sports injuries, all of which aim at bringing injured athletes to the highest levels of performance as quickly as possible and to prolong the professional life of elite athletes.

The use of various types of stem cells in patients and athletes has been discussed and revealed that the future will be a witness for the use of multiplying autologous stem cells as a therapeutic option for musculoskeletal disputes such as arthritis and cartilage defects. Stem cell therapy is a progressive and relatively safe process for the treatment of many diseases. It will be necessary to continue studies in the field of stem cell therapy for sports injuries to better understand the capabilities of individual cells to treat pain and improve injury healing. Professional athletes have previously been deprived of this treatment option, and in recent years they have

sometimes gone abroad to use this type of treatment. The use of cell therapy for patients when the hockey player legend was treated with stem cell attracted more international attention. Athletes, whether they are still in exercise or retired, have a strong need for stem cell regeneration ability, due to severe injuries such as bone fractures, ligament dilatations, and cartilage wear, which have been experienced during exercise.

Perhaps the sports medicine developments were divided into three periods in the past, present, and future. If, in the past few years, the presence of a physician or a medical practitioner alongside a sports ground with equipment such as kneading and cooling spray was a symbol of progress, by presenting the field of sports medicine as one of the fields of medical specialty, the field for the provision of services, as well as using the abovementioned advancements in the management of professional athletes, is more than ever possible; however, there are still many challenges and resistances against the entry of sports medicine professionals in the field of professional sport.

We hope that this promising method leads to increasing and improving the laboratory aspects of using these cells readily available to all patients, globally. In the near future, it seems that the role of sports medicine is also highlighted and strategies such as focusing on prevention and identification of risk factors of injury, therapeutic use of stem cells, traumatic sports injuries, and genetic manipulation by completing the human genomic project become more operational.

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Stem Cell Therapy for Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory, autoimmune, and neurodegenerative disease of the central nervous system (CNS). It is characterized by demyelination and neuronal loss that is induced by attack of autoreactive T cells to the myelin sheath and endogenous remyelination failure, eventually leading to functional neurological disability. Although recent evidence suggests that MS relapses are induced by environmental and exogenous triggers such as viral infections in a genetic background, its very complex pathogenesis is not completely understood. Therefore, the efficiency of current immunosuppression-based therapies of MS is too low, and emerging disease-modifying immunomodulatory agents such as fingolimod and dimethyl fumarate cannot stop progressive neurodegenerative process. Thus, the cell replacement therapy approach that aims to overcome neuronal cell loss and remyelination failure and to increase endogenous myelin repair capacity is considered as an alternative treatment option. A wide variety of preclinical studies, using experimental autoimmune encephalomyelitis model of MS, have recently shown that grafted cells with different origins including mesenchymal stem cells (MSCs), neural precursor and stem cells, and induced-pluripotent stem cells have the ability to repair CNS lesions and to recover functional neurological deficits. The results of ongoing autologous hematopoietic stem cell therapy studies, with the advantage of peripheral administration to the patients, have suggested that cell replacement therapy is also a feasible option for immunomodulatory treatment of MS. In this chapter, we overview cell sources and applications of the stem cell therapy for treatment of MS. We also discuss challenges including those associated with administration route, immune responses to grafted cells, integration of these cells to existing neural circuits, and risk of tumor growth. Finally, future prospects of stem cell therapy for MS are addressed.

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Keywords
Experimental autoimmune encephalomyelitis · Hematopoietic stem cell · Induced pluripotent stem cell · Mesenchymal stem cell · Multiple sclerosis · Neural stem cell · Reprogramming · Stem cell therapy

Abbreviations

AD-MSCs	Adipose tissue-derived MSCs
AHSCT	Autologous hematopoietic stem cell transplantation
APC	Antigen-presenting cells
ASC	Adult stem cells
BBB	Blood–brain barrier
CNS	Central nervous system
Cy	Cyclophosphamide
DC	Dendritic cells
DMDs	Disease-modifying drugs
Dpi	Days of post immunization
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale
ESC	Embryonic stem cells
G-CSF	Granulocyte colony-stimulating factor
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine 2,3-dioxygenase
IFNɣ	Interferon gamma
IL-10	Interleukin-10
IL-1β	Interleukin-1beta
iNSC	Induced neural stem cell
iOL	Induced oligodendrocyte
iOPC	Induced oligodendrocyte progenitor cell
iPSC	Induced pluripotent stem cell
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
NK	Natural killer
NPC	Neural progenitor cells
NSC	Neural stem cell
OPC	Oligodendrocyte progenitor cell
PBMC	Peripheral blood mononuclear cells
PMS	Progressive MS
RRMS	Relapsing-remitting multiple sclerosis
SCT	Stem cell transplantation
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
SVZ	Subventricular zone
Th	T helper
TNF	Tumor necrosis factor
Tregs	T cell regulatory
TRM	Transplantation related mortality

1 Multiple Sclerosis

1.1 Overview of Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating, and autoimmune disease of the central nervous system (CNS). Myelin sheaths of neurons are attacked by autoreactive T and B cells, specific to myelin autoantigens such as myelin basic protein (MBP). MS was described in 1868 by Jean-Martin Charcot who observed multiple lesions and glial scar (plaque) areas in the white matter of the brain and medulla spinalis Gomes Mda and Engelhardt 2013). MS is characterized with segmental demyelination, axonal injury, and neuron and oligodendrocyte loss leading to neurological dysfunction and disability (Kawachi and Lassmann 2017). MS affects approximately 2.5 million people worldwide. High prevalence of MS is seen in northern parts of Europe and North America (Browne et al. 2014). MS is a major economical and social burden for modern societies, because of its progressive, chronic, and debilitating nature, lack of any cure, and its target age group, who are young adults, the most productive members of the population.

1.2 Etiology

The main cause of MS is not fully understood, but several genetic and environmental factors such as smoking contribute to the development of disease (Olsson et al. 2017). MS is not considered a hereditary disease; however genome-wide association studies (GWAS) have shown that several single nucleotide polymorphisms (SNPs) in immune system-related genes associate with predisposition to MS. Immune-related gene variants, of both adaptive and innate systems, are associated with MS (Parnell and Booth 2017). It has been confirmed by different studies that the human leukocyte antigen (HLA) *DRB1*1501* is associated with MS susceptibility in many populations (Olsson et al. 2017). Various infectious agents, especially Epstein-Barr virus, have been suspected in the etiology of MS for over a century (Mentis et al. 2017).

1.3 Clinical Presentation of Multiple Sclerosis

The specific signs and symptoms of MS are dependent on the neuroanatomical localization of the lesions in the CNS. Typical signs and symptoms include optic neuritis, diplopia, muscle weakness, sensory deficits, and ataxia (Compston and Coles 2008). Cognitive, behavioral, and emotional problems are also commonly seen in later stage of the disease. Clinical course of MS is categorized into four subtypes. "Clinically isolated syndrome" is the first episode of neurologic symptoms lasting at least 24 h that are suggestive of MS (Tsang and Macdonell 2011).

Clinically isolated syndrome (CIS) refers to a single clinical attack of central nervous system (CNS) inflammatory demyelinating symptoms that are suggestive of multiple sclerosis (MS).

"Relapsing-remitting multiple sclerosis" (RRMS) is the most common form of MS that is characterized by recurrent attacks or increasing neurologic symptoms and are followed by remission periods (Lublin et al. 2014). After 20–25 years, 90% of patients with RRMS turn into "secondary progressive multiple sclerosis" (SPMS) which is characterized by progressive neurological decline without relapses. Approximately 10–15% of patients with MS are diagnosed with "primary progressive multiple sclerosis" (PPMS) which is characterized by the steady progressive worsening neurologic function from the onset of symptoms, without relapses (Lublin et al. 2014). Some MS patients show rapid progression in a very short time period, and this particular type is called "aggressive MS" (Rush et al. 2015). To know the course and type of MS is essential to predict prognosis and to make treatment decisions both in DMTs and stem cell therapies.

1.4 Diagnosis of MS

The diagnosis of MS is based on neurologic findings supported by magnetic resonance imaging (MRI), cerebrospinal fluid, and evoked potential analyses (Garg and Smith 2015). The most recently revised McDonald criteria allowed safe and early diagnosis of MS (Polman et al. 2011). MRI has rapidly become a leading diagnostic tool in MS. Brain MRI shows T2 hyperintense white matter lesions in periventricular, juxtacortical, and infratentorial regions (Filippi et al. 2017b). Dissemination of MRI lesions in time and space are critically important for MS diagnosis.

1.5 Histopathology

Pathological hallmarks of MS are inflammation, gliosis, demyelination, axonal injury, and synaptic loss (Garg and Smith 2015). The MS plaques are localized demyelination areas with different degrees of inflammatory cell infiltrations predominantly located in the white matter of the brain, spinal cord, and optic nerves (Ransohoff et al. 2015). Demyelinated plaques can also locate in the cortical and subcortical gray matter. The inflammatory infiltrates are composed of activated T cells, activated macrophages/microglia, plasma cells, and B cells. While active plaques are characterized by myelin degradation,

reactive astrocytes, and perivascular and parenchymal inflammation, more extensive demyelination, axonal loss, oligodendrocyte injury, and less active inflammation are observed in chronic plaques (Popescu et al. 2013). Blood-brain barrier (BBB) disruption is another pathological feature of MS and allows immune cell infiltration into the brain. Remyelination following acute inflammatory episodes contributes to functional recovery, but it is frequently insufficient to restore neurological functions. The cortical brain atrophy is a characteristic histopathological feature of MS and is associated with cognitive impairment in progressive MS (PMS) (Filippi et al. 2017b).

1.6 Pathogenesis

Although the pathogenic factors and mechanisms which lead to MS are largely unknown, the current view is that environmental triggers such as infection initiate progress of disease in genetically predisposed individuals. A wide variety of genetic, clinical, pathological, and epidemiological studies support this hypothesis. In addition to the well-known HLA-DRB1 risk gene, GWAS studies showed presence of MS-associated SNPs in non-MHC immune regulatory genes that are involved both in innate and adaptive immunity in MS patients (Parnell and Booth 2017). Interestingly, MS-associated gene loci show little overlap with those of primary neurodegenerative diseases (Hemmer et al. 2015). In contrast, one-third of disease-associated risk genes are common in MS and other autoimmune diseases (Parnell and Booth 2017; Yadav et al. 2015). Both brain biopsy and postmortem autopsy studies show immune cell infiltration in MS brain. Histopathological investigations confirm this finding in animal models of MS. Another evidence suggesting that immune component plays an important role in MS pathogenesis is the recovery of MS attacks by immunosuppressive and immunomodulatory therapies (Yadav et al. 2015). Finally, immunological analyses performed with body fluids and peripheral blood mononuclear cells (PBMC) samples of MS patients show aberrant immune parameters.

In healthy individuals, the immune system plays a vital role in self-defense mechanisms against bacteria, virus, and other environmental hazardous factors, discriminating self and non--self-antigens. Following the removal of foreign antigens, immune homeostatic mechanisms provide the resolution of inflammation and fading of immune responses. Self-antigen recognizing autoreactive T cells are deleted in thymus. Several circulating autoreactive T cells that escaped from thymic deletion are suppressed by regulatory T cells (Tregs) and normally do not encounter myelin antigens because of BBB (Jones and Hawiger 2017). It is thought that Treg activity and proliferation decrease and/or autoreactive T cells resist immunoregulatory mechanisms in MS. Both cellular and humoral components of adaptive and innate immunity are involved in autoimmune responses that mediate neurotoxic and gliotoxic injury.

Either bacterial or viral infections frequently trigger MS attacks. Myeloid cells of innate immunity (dendritic cells, macrophages, and microglia) become activated and mature when pathogen-associated molecular patterns such as bacterial lipopolysaccharide or viral antigens bind to pattern recognition receptors including Toll-like receptors and NOD-like receptors. Upon activation, these cells secrete interleukin-1beta (IL-1β) and interleukin-18 (IL-18) cytokines that induce expression of chemokines and their receptors both by T cells and antigen-presenting cells (APC), enhancing T cell migration (Lin and Edelson 2017).

Some other cytokines secreted by mature dendritic cells (DC) polarize CD4+ T helper (Th) subtypes. IL-1β and interleukin-23 stimulate Th17 cells that secrete interleukin-17 (IL-17). Interleukin-12 induces interferon gamma (IFNγ)-secreting proinflammatory Th1 cells (Grigoriadis et al. 2015). DCs are also APCs and using MHC-II receptors recognize self- or non-self-antigens that show structural similarity to myelin antigens. The second mechanism is called molecular mimicry that can initiate autoimmune responses. CNS antigens can pass to systemic circulation through glymphatic drainage system and are presented to CD4+ T cells by

DCs in deep cervical lymph nodes (Simon and Iliff 2016). Th1 and Th17 cells disrupt the structure of BBB and increase its permeability by cytokines, chemokines, and matrix metalloproteinases. These soluble factors also mediate the recruitment and infiltration of other immune cells (macrophages, B cells, CD8+ T cells, and natural killer (NK) cells) to the CNS (Dargahi et al. 2017). All of these cells and their products lead to immune-mediated glial and neuronal injury and cell death. Upon activation, B lymphocytes convert to autoantibody-secreting plasma cells. Myelin-reactive autoantibodies result in myelin injury and antibody- and complement-dependent death of oligodendrocytes (OLs) and neurons (Compston and Coles 2008; Disanto et al. 2012). Recruited CD8+ T cells bind to MHC-I receptor-expressing cells including oligodendrocytes and neurons and secrete cytotoxic granules containing perforin and granzyme B that lyse cells by pore formation (Salou et al. 2015). Activated T cells can also kill oligodendrocytes or neurons through Fas-Fas ligand and tumor necrosis factor (TNF)-α-TNF-α receptor interactions (Connick et al. 2012). Autoreactive T cells activate microglia and polarize them to M1 proinflammatory phenotype by TNF-α and IFNɣ (Yadav et al. 2015). Microglia can phagocytose oligodendrocytes or kill these cells indirectly by soluble factors including nitric oxide, reactive oxygen species, reactive nitrogen species, glutamate, and proinflammatory cytokines (Kawachi and Lassmann 2017). Liberation of iron from dead oligodendrocytes amplifies oxidative injury (Kawachi and Lassmann 2017). Autoregulatory immune mechanisms move in to resolve inflammation in acute MS attacks. Inflammation-resolving lipid mediators such as resolvin D, anti-inflammatory cytokines such as interleukin-10 (IL-10), Treg cells and microglia which are polarized to M2 phenotype, and neurotrophic factors secreted from glial cells control autoimmune responses and repair damaged CNS tissue (Guo 2016; Miron 2017).

In contrast, uncontrolled autoimmune responses amplify and increase tissue damage in chronic phase. Activated microglia are predominant in cerebral parenchyma, and B cells constitute meningeal ectopic foci. Main property of chronic neuroinflammation in MS is its compartmentalization.

1.7 Current Treatment

There is no cure for MS. Currently available treatments for MS help improve patient's overall quality of life and minimize long-term disability by preventing the frequency of relapses and severity of acute MS attacks (Garg and Smith 2015). In recent years, many new disease-modifying drug (DMD) options have become available. These drugs primarily target the underlying immunologic etiology of the disease. Interferon-ß and glatiramer acetate have been used as first-line DMDs for RRMS over two decades (Comi et al. 2017). Teriflunomide, fingolimod, and dimethyl fumarate are other moderately effective immunomodulators. Teriflunomide and fingolimod suppress activated T and B lymphocytes through different mechanisms (Grigoriadis et al. 2015). Natalizumab specifically inhibits cell migration via integrin blockage (Delbue et al. 2017). Alemtuzumab, which is newly introduced in MS treatment, reduces CD52+ T and B cells. While these drugs significantly reduce the frequency and severity of MS attacks, they have serious side effects including progressive multifocal leukoencephalopathy, hypertension, leukemia, viral infections, teratogenesis, and cardiac arrhythmias (Comi et al. 2017). These adverse effects hinder their long-term use. They also have limited long-term effectiveness, high cost, and inability to reverse disease (Dargahi et al. 2017). Eventually the majority of RRMS patients turn to progressive MS. DMDs do not prevent progressive neurodegenerative processes. Thus, new drugs and treatment regimens are required to effectively treat both primary and secondary PMS (Dargahi et al. 2017). As MS enters progressive phase, inflammation continues, but it evolves to a more CNS-restricted pattern without BBB leakage (Grigoriadis et al. 2015). Hence, new CNS-targeted immunomodulatory drugs that can

cross repaired BBB and reach to ectopic B cell follicles are still needed.

However, DMDs in multiple sclerosis target the immune system and do not have regenerative effect. Regenerative treatment approaches promoting remyelination are promising for MS treatment (Plemel et al. 2017).

2 Basics of Stem Cell Biology

Stem cells are undifferentiated cells that are capable of self-renewal and have the ability to give rise to specialized cells through asymmetric division (Tabansky and Stern 2016). Stem cells are categorized into two main parts: embryonic stem cells and adult stem cells.

Embryonic stem cells (ESC) give rise to all cell types and differentiate into tissue types apart from extraembryonic tissues (Tabansky and Stern 2016). This feature is defined as pluripotency. Furthermore, they are able to pass unlimited and symmetric mitotic divisions without being specialized. ESCs are derived from blastocyst, which is a premature embryo that forms 5 days after fertilization. ESCs are obtained from the blastocyst's inner cell mass that consists of 30 cells (Tabansky and Stern 2016). Zygote and the cells at very early stages of fertilization are defined as totipotent (Wu et al. 2016). They have ability to constitute all the cells needed to generate a complete organism. Usage of ESCs for research has started to create ethical controversies because blastocyst is destroyed and loses its ability to generate a human (King and Perrin 2014).

Adult stem cells (ASCs) differentiate into limited types of cell of tissue or organ where they are located (Mariano et al. 2015). They are characterized as multipotent cells because of their capacity to produce tissue-specific cell types. Their main function is to repair or produce specialized cells when wear and tear, injury, or disease occurs. ASCs have been found in many different tissues or organs and are divided into categories depending upon their locations or diversified features such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and neural stem cells (NSCs). HSCs can be found in the bone marrow (BM), peripheral blood, and umbilical cord blood and generate all types of blood cells (Ng and Alexander 2017). MSCs are also located in the BM and develop into different types of cells, including fat cells, cartilage, bone, tendon and ligaments, muscles cells, skin cells, and even nerve cells (Volkman and Offen 2017). NSCs are located in the brain and constitute neurons and glial cells (Nam et al. 2015). ASCs' isolation is very hard and impractical. In 2006, Yamanaka and his co-workers found a solution to this problem by converting somatic cells into pluripotent stem cells (Takahashi and Yamanaka 2006). These reprogrammed pluripotent stem cells are called as induced pluripotent stem cells (iPSCs). iPSCs have similar features to embryonic stem cells, such as giving rise to many varied tissue types, and are able to divide indefinitely in culture. However, the risk of tumor formation of iPSCs is very high (Heslop et al. 2015). This risk is reduced by a procedure called transdifferentiation (Cieslar-Pobuda et al. 2017), in which a cell type is converted into another cell type in different tissues without going through a pluripotent cell state. Progenitor cells such as induced neural stem cells (iNSC), induced oligodendrocyte progenitor cells (iOPC), and induced oligodendrocytes (iOL) can be derived from a somatic cell type by transdifferentiation (An et al. 2016).

3 Hematopoietic Stem Cell Therapy

Hematopoietic stem cells are rare cells with characteristics of pluripotentiality and self-renewal ability and constitute about 0.01% all total nucleated cells in the BM. HSCs are capable of generating all hematopoietic cell lineages including erythrocytes, megakaryocytes, platelets, and innate and adaptive immune system cells (Ng and Alexander 2017). HSCs undergo self-renewal when transplanted to humans and are able to differentiate into all of the hematopoietic cell types. HSC transplantation (HSCT) has been used for about half a century in the clinic as an

effective therapeutic approach in cancer. In the early 1990s, preclinical studies showed that HSCT is also effective in various experimental models of autoimmunity including experimental autoimmune encephalomyelitis (EAE) (Karussis and Slavin 2004). During the last two decades, HSCT became an alternative therapy option to immunosuppressive and immunomodulatory drugs in autoimmune diseases such as MS. Here, the rational for HSCT is based on the concept of rebooting the aberrant immune system through the elimination of autoantigen-reactive T and B lymphocytes, increase of Treg population, and reconstitution of self-tolerance (Swart et al. 2017).

3.1 Procedure

HSCs are mobilized from BM by treatment with cyclophosphamide (Cy) and granulocyte colony-stimulating factor (G-CSF). The reason of Cy administration is to prevent a possible MS relapse due to G-CSF (Bell et al. 2017). After 4 or 5 days, HSCs are collected by peripheral vein leukapheresis. Following staining by anti-CD34 monoclonal antibody, cells are purified using either fluorescence-activated cell sorting or magnetic-activated separation and then are stored frozen until transplantation. The selection of CD34+ cells increases the purity excluding autoreactive lymphocytes. The minimum number required for autologous HSCT (AHSCT) is 3×10^6 CD34+ cell/kg/body weight. After 4 or 5 weeks, the thawed AHSCT are reinfused to the patient. Generally, 3–5% of all the cells in a graft are HSCs (Atkins and Freedman 2013). To prevent the expansion of autoreactive lymphocytes after transplantation, immune ablative conditioning regimens that consist of chemotherapeutics and immunosuppressive drugs are used. The doses and combinations of conditioning drugs determine the intensity of regimens which are positively correlated with the outcome of AHSCT, the frequency of side effects, and transplantation-related mortality (TRM). TRM is percentage of mortality in the first 100 days after transplantation. The most widely conditioned scheme is intermediate-intensity regimen which is called BEAM and includes BCNU, etoposide, AraC, and melphalan. Low-intensity regimens are used to reduce the toxicity related to intense immunosuppression. The conditioning regimen is followed by infusion of autologous CD34+ stem cells. Most patients are lymphopenic during several months after AHSCT while their immune system fully reconstitutes (Sarkar et al. 2017). Prophylactic acyclovir treatment is used for 1 year posttransplantation to prevent viral infections (Bell et al. 2017).

3.2 Clinical Studies

AHSCT has been applied to MS patients since 1997 (Fassas et al. 1997). The results of early clinical studies with MS patients who underwent AHSCT vary due to small sample sizes, different cohort characteristics, included populations with different proportions of RRMS and PMS, distinct conditioning regimes for AHSCT, and diverse toxic effects of different drugs, which make comparisons between studies difficult (Dorr 2016). The more recent clinical AHSCT studies in RRMS have reported beneficial effects based on Expanded Disability Status Scale (EDSS) score and MRI activity (Burman et al. 2014; Burt et al. 2015; Nash et al. 2015), with no TRM. In a more recent study, AHSCT without maintenance immunosuppressive therapy was effective for inducing long-term sustained remission of active RRMS at 5 years follow-up (Muraro et al. 2017). The only completed controlled randomized clinical study is the *A*utologous *S*tem *C*ell *T*ransplantation *I*nternational *M*ultiple *S*clerosis (ASTIMS) trial (Mancardi et al. 2015). This study, comparing mitoxantrone versus AHSCT, had both aggressive RRMS and SPMS patients. Although no difference was observed in EDSS score, results showed that AHSCT is superior with regard to MRI activity and relapse rate.

AHSCT is not effective in PMS despite aggressive immune ablation regime resulting in a posttransplant immune reset (Casanova et al. 2017). Neurological disability observed in

SPMS is mainly caused by neurodegenerative processes due to axonal atrophy and not inflammatory process. As a result, the progressive phase may not be treatable by neither immunomodulatory agents nor AHSCT.

3.3 Patient Selection Criteria for Autologous Hematopoietic Stem Cell Transplantation

Good candidates for AHSCT are patients in early phase of disease. The patient inclusion criteria for AHSCT in MS are as follows: RRMS, age between 18 and 45 years, duration of MS not exceeding 5 years, EDSS between 2.5 and 6.5 points, clinically active disease, and evidence of gadolinium enhancement on MRI (Bell et al. 2017).

3.4 Follow–Up and Outcome

Long-term follow-up (over years) is mandatory. In a recent long-term outcome study, Muraro et al. have reported that factors associated with neurological progression after AHSCT are older age, PMS form of MS, and more than two previous DMTs (Muraro et al. 2017) .

"No evidence of disease activity (NEDA)" status has emerged as a composite measure of RRMS treatment success and is defined as "no evidence of relapse," "no evidence of disability progression," and "no MRI activity, namely absence of new or enlarging T2 lesions or Gd-enhancing lesions" (Matta et al. 2016). NEDA is used as a primary endpoint in AHSCT. A recent meta-analysis study has reported that pooled proportion of NEDA patients in AHSCT was 83% (70%–92%) at 2 years and also was 67% (59%–70%) at 5 years (Sormani et al. 2017). Favorable outcome was seen in young patients with clinical and radiologically active disease, having short disease duration.

3.5 Parameters of Treatment Effectiveness

The parameters of treatment effectiveness are relapse-free survival, MRI event-free survival, and progression-free survival (Burman et al. 2014). Disease-free survival is determined by absence of both clinical and radiological disease sign and symptoms in a particular period. Sustained reduction in disability is defined as the improvement of 1.0 point in the EDSS score sustained 6 months.

3.6 Biomarkers

The combination of specific and selective biomarkers will contribute to a better and earlier selection of patients for ASHCT and follow-up process (Londono and Mora 2016). It is important to identify these patients using putative predictive markers as early as possible and to apply effective treatment strategies. Unfortunately, there are no predictive markers to identify patients who will develop aggressive MS.

3.7 Safety Profile and Adverse Effects

Early side effects of ASHCT are fever and viral infection, while a late adverse effect is the development of autoimmune thyroiditis (Zeher et al. 2017). Following AHSCT, some MS patients may show accelerated brain atrophy that is likely associated with busulfan neurotoxicity and neurodegeneration of committed tissues (Lee et al. 2017). The rate of determined brain atrophy declines to that of expected for age-matched healthy people. The same group found that busulfan dose was a significant predictor of white matter and gray matter atrophy. Long-term rates of gray and white matter atrophy were comparable to those of healthy controls at the same age. Cryopreservation is recommended against infertility (Bell et al. 2017).

The overall TRM (1995–2106) for AHSCT is 2%, and it has decreased to 0.2% over the last 5 years (2012–2016) (Muraro et al. 2017). Nowadays, TRM reaches 0% in several centers, which may result from standardized optimal transplant procedures being implemented and presence of effective collaboration between transplant hematologists and neurologists.

3.8 Immune Mechanisms

The exact mechanisms of AHSCT's therapeutic effect in RRMS are not fully understood. The early effect is instant and temporary radical depletion of autoreactive pathogenic immune cells due to ablative conditioning regimes. The use of AGT in conditioning regimen specifically may also play a role through complement-mediated lysis of autoantibody-producing plasma cells (Zand et al. 2005). In the late phase, adaptive immune system is reconstituted from newly formed naïve T and B cells by increased thymopoietic activity. Naïve B cell reconstitution restores the B cell repertoire and antibody diversity. Following AHSCT, Th1 and Th17 activities apparently decrease (Karnell et al. 2017). Clonal diversity of T cell receptor (TCR) improves, and subsequently new and diverse T cell repertoire develops (Muraro et al. 2005). After AHSCT, diverse immune cells are repopulated in a particular order. The immune repopulation process has very special dynamics. Firstly, innate immune system cells appear just within weeks following AHSCT. Monocytes are the first cells to engraft with subsequent population by neutrophils and NK cells. While CD8+ T lymphocytes return to normal values 3 months after transplantation, B lymphocytes reach a normal value at 6 months (Zeher et al. 2017).

Treg cells erase and suppress proinflammatory and autoimmune processes driven by Th17 cells and decrease the development of autoreactive T cell clones (Zeher et al. 2017). Restoration of gene expression changes takes longer time after AHSCT (Muraro et al. 2017).

Not all patients receiving autologous HSCT have achieved long-term clinical responses (Kelsey et al. 2016). When patients cannot develop a diverse repertoire of naive T cells after AHSCT, their response to treatment is likely to be less successful (Muraro et al. 2014). Further studies are necessary to determine which immune mechanisms contribute to the effect of AHSCT in MS (Muraro et al. 2017).

3.9 Cost-Effectiveness and Risk-Benefit Ratio of AHSCT

Risk-benefit profile and cost-effectiveness of AHSCT are comparable with those of current MS drugs. These drugs may lead to serious adverse effects such as PML (natalizumab), secondary autoimmune disease (alemtuzumab), and cardiac arrhythmias (fingolimod) (Bell et al. 2017; Burt et al. 2012; Curro and Mancardi 2016). Furthermore, these medications are expensive and are usually continued infinitely or until complications arise. In comparison, patients' compliance to HSCT is high (Burt et al. 2012).

3.10 Future Studies and Prospects

Well-designed randomized controlled studies that systematically investigated the effect and safety of HSCT compared to other relevant treatments are needed in order to clarify treatment benefits. Two randomized controlled phase III trials are being conducted to compare AHSCT with FDA-approved DMDs (MIST Study [ClinicalTrials.gov identifier: NCT00273364 and NCT03133403]. iPSCs and transdifferentiated somatic cells have been proposed as an alternative source of HSCs for possible applications that include autologous, autologous and genetically modified, or allogeneic cells (van Bekkum and Mikkers 2012). Currently, transplantation of iPSC-derived hematopoietic cells is still limited to preclinical animal models. Low hematopoietic differentiation efficiency and tumor formation risk must be overcome for clinical application (Hwang et al. 2017). If pathogenic gene variants are detected in MS patients, iPSC or transdifferentiated cells derived from differentiated somatic

cells can be genetically corrected using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 gene editing and converted into normal hematopoietic cells that can be used in AHSCT.

4 Mesenchymal Stem Cell Therapy

4.1 Biology of Mesenchymal Stem Cells

MSCs are self-replicating cells which were first described by Friedenstein et al. in 1968 (Friedenstein et al. 1974). They were first isolated from BM; besides BM a wide range of adult tissues including adipose tissue, umbilical cord blood, placenta, and dental pulp have been used as sources of MSCs (Gharibi et al. 2015; Giacoppo et al. 2017). There is no specific marker to discriminate MSCs from other cells. The minimum criteria for MSCs determined by the International Society for Cell and Gene Therapy are plastic adherence; presence of CD105, CD73, and CD90 expression; absence of hematopoietic surface markers (CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-D); and differentiation capacity to osteoblasts, adipocytes, and chondroblasts in vitro (Dominici et al. 2006; Sarkar et al. 2017).

4.2 In Vivo Studies

In vivo studies investigating the effect of MSCs on EAE have been started after demonstration of their suppressive effects on T cell proliferation in vitro and in vivo. MSCs derived from several different adult tissues were used in acute and chronic MS animal models via different routes (Giacoppo et al. 2017; Sarkar et al. 2017). An early EAE study showed that administration of murine BM-derived MSCs at the early stage of disease reduced clinical scores, demyelination, and inflammatory infiltrates in the CNS (Zappia et al. 2005). MSC transplantation also ameliorated proteolipid protein (PLP)-induced EAE symptoms in SJL/J mice through the inhibition of pathogenic T and B cell responses (Gerdoni et al. 2007). Human BM-derived MSCs promote clinical recovery in chronic and relapsing-remitting mouse models of MS, possibly via reduced Th1 and Th17 cells and increased IL-4-producing Th2 cells (Bai et al. 2009). Various studies reported therapeutic effects of adipose tissue-derived MSCs (AD-MSCs) in EAE (Giacoppo et al. 2017; Li et al. 2017; Scruggs et al. 2013; Semon et al. 2014; Strong et al. 2016). Intraperitoneal administration of AD-MSCs provides more Treg cells and IL-4 production than intravenous route (Yousefi et al. 2013). Age and body mass index of donors alter therapeutic effect of MSCs. MSCs derived from older donors are less effective than younger donors in myelin oligodendrocyte glycoprotein (MOG)-induced EAE (Scruggs et al. 2013). AD-MSCs from obese donors could not suppress clinical signs of EAE and inflammation in the brain (Strong et al. 2016). As autologous AD-MSCs had no therapeutic effect on the EAE progression (Semon et al. 2014), allogenic AD-MSC treatment may be preferred in clinical trials of MS. Fetal tissues including the placenta, amnion epithelial cells, umbilical cord, umbilical cord matrix, and decidua have been used for MSC generation. These fetal tissue-derived MSCs ameliorate EAE disease severity and inflammation of CNS (Giacoppo et al. 2017). Fetal tissues contain large number genetically stable stem cells, so they have become candidate alternative tissue sources for the MSCs. However, these favorable results of MSC transplantation in EAE may not necessarily indicate that MSC treatment will be effective in clinical studies. Because, animal models of EAE do not reflect all aspects of MS and human MSCs have special features that differ them from mouse stem cells.

4.3 Clinical Studies

In an initial pilot human study, autologous MSC treatment was carried out in ten PMS patients via intrathecal route (Mohyeddin Bonab et al. 2007). Mild improvement in clinical sign of MS was

observed in a 13–26-month follow-up period. Several studies in small patient groups have provided evidence on safety and efficacy of a single-dose MSC treatment (Table 1) (Bonab et al. 2012; Cohen et al. 2017; Connick et al. 2012; Karussis et al. 2010; Llufriu et al. 2014; Mohajeri et al. 2011; Mohyeddin Bonab et al. 2013; Yamout et al. 2010). Bonab et al. reported that improvement or stabilization of clinical or MRI findings was seen in 15 of 25 patients (Bonab et al. 2012; Mohyeddin Bonab et al. 2013). Their results support that MSC therapy will be effective in unresponsive MS cases. Intrathecal administration of MSCs did not alter cytokine expression in peripheral blood (Mohyeddin Bonab et al. 2013), but increased Treg cells and suppressed the proliferative responses of lymphocytes, and the expression of CD40+, CD83+, CD86+, and HLA-DR+ myeloid DCs (Karussis et al. 2010). Repeated intravenous infusions of autologous MSCs every month during 4–8 months resulted in clinical improvement in EDSS scores in six of eight patients (Odinak et al. 2011). BM-derived MSCs were converted to neural progenitor cells (NPCs) and administrated to eight PMS patients via intrathecal route. Two to five repetitive treatments of MSCs provided improvement of clinical sign in four of eight patients. Repeated intrathecal administrations of MSC-derived NSCs were well-tolerated by six PMS patients in both short-term and long-term periods (7.4 years) (Harris et al. 2016). No serious adverse events were reported during follow-up periods of clinical MSC transplantation studies. Mild self-limited adverse events, such as headache, fever, nausea, vomiting, and weakness in the lower limbs, have been observed.

Apart from these published studies, several clinical trials for MSC treatment in MS are ongoing. A phase II open-label clinical trial for BM-derived MSC in PMS will include 80 patients, whose results are awaited (ACTiMuS) (ClinicalTrials.gov (NCT01815632)). A phase III MSC transplantation study was terminated due to limited number of participation (CMM-EM) (ClinicalTrials.gov (NCT01228266)). There are two AD-MSC transplantation clinical trials in MS listed in ClinicalTrials.gov (NCT01056471 and NCT02326935). A phase I/II randomized placebo-controlled study evaluating safety and feasibility of therapy of autologous MSCs in patients with SPMS was completed (NCT01056471). The other phase I multicenter study is currently recruiting patients (NCT02326935). The results of these two clinical trials are expected.

4.4 Mechanisms of Action

MSCs have immunomodulatory, immunosuppressive, neurotrophic, and repair functions (Giacoppo et al. 2017; (Sarkar et al. 2017). They inhibit both innate and adaptive immune responses. Decreased proliferation and immune responses of T cells, B cells, NK cells, and APC are observed (Gharibi et al. 2015). BM-derived MSCs have transdifferentiation capacity into neuron-like cells in vitro under certain conditions (Uccelli et al. 2011), but cell replacement effect of MSCs was not observed in EAE and clinical studies. Soluble factors secreted by MSCs and cell-to-cell contact have been the implicated mechanisms of MSCs' effects. Inoleamine-2,3-dioxygenase, transforming growth factor-β, hepatocyte growth factor (HGF), nitric oxide, and soluble HLA-G are soluble factors which mediate MSCs therapeutic effects (Bai et al. 2012; Mahfouz et al. 2017; Matysiak et al. 2008).

4.5 Challenges and Future Studies

There are several safety issues with MSC transplantation including infusion-related toxicity, infection, malignancy development, and disease activation. Standard procedures about dose, route of administration, repetition time, and culture conditions for MSC treatment in MS should be developed. The unsolved problems for MSC transplantation in MS are to provide migration of cells to lesion site and homing of cells into donor tissue. To avoid side effects of cell therapy, administration of MSC-derived exosomes could be a noncellular alternative therapy option to stem cell transplantation (SCT) in MS (Jarmalaviciute and Pivoriunas 2016). Recently, good

Table 1 Clinical studies with MSCs in multiple sclerosis

Author	Trial	Folow-up	MS type	N (F/M)	Age	EDSS	Transplanted cell	Route of administration	Single/ repeated	Outcomes	Side effects
Bonab 2007		13–26 months	PMS	10		3.5–6	Autologous BM-MSCs	IT	Single	EDSS of one patient improved from 5 to 2.5; four patients no change; five patients increased from 0.5 to 2.5 MRI: seven patients with no difference, two extra plaque, and one patient decrease in the number of plaques	
Karussis 2010	I/II	6–25 months	N/A	15 (8/7)	35.3	6.7 (4–8)	Autologous BM-MSCs	IT and IV	Single	EDSS score improved from 6.7 (1.0) to 5.9 (1.6) No new or Gd + lesions An increase in the proportion of Treg cells, decrease in proliferative responses of lymphocytes, and expression of CD40+, CD83+, CD86+, and HLADR on myeloid DCs	Mild self-limited febrile reaction, headache
Yamout 2010	Pilot	12 months	SP RR	7 (4/3)	42	6.42 (4–7.5)	Autologous BM-MSCs	IT	Single	EDSS improvement in 5/7, stabilization in 1/7, and worsening in 1/7 patients. MRI: a new or enlarging lesion in 5/7 and Gd + lesions in 3/7 patients. Vision and low-contrast sensitivity testing: improvement in 5/6	Transient encephalopathy with seizure in one patients
Mohajeri 2011		6 months	RR	7 (6/1)	35.5	N/A	Autologous BM-MSCs	IT	Single	FOXP3 mRNA increased	

Connick 2012	IIA	6 months	SP	10 (3/7)	48.8	N/A (2.0–6.5)	Autologous BM-MSCs	IV	Single	Improvement after treatment in visual acuity and visual evoked response latency, increase in optic nerve area	No serious adverse events
Bonab 2012, 2013		12 months	PP SP	25 (19/6)	34.7 (23–50)	5.5–7	Autologous	IT	Single	MRI (3 T) and EDSS scores improved or remained unchanged in 15 (68.18%) patients, whereas 7 (31.81%) patients showed new T2 or Gd + lesions or increased EDSS. Three (13.63%) patients refused to undergo further follow-up after 6 months	Transient low-grade fever, nausea/vomiting, weakness in the lower limbs, and headache
							BM-MSCs			No changes in gene expression and serum level of cytokines	
Odinak 2012		4–12 months	RR PPSP	8 (3/5)	37.5 (24–47)	5.56 (3.5–6.5)	Autologous BM-MSCs	IV	Repeated per month, 4–8 months	Improvements on the EDSS by 0.5–1 point were seen in six of the eight patients, with stabilization in one and progression in another patient	No serious adverse events. General weakness
Lilufru 2014	II	6 months	RR	9 (7/2)	36.8	3.5 (3.0–6.0)	5 patient BM-MSCs; 4 patient placebo	IV	single	No clinical significant change, nonsignificant reduction in Gd + MRI lesions and Th1 cells	No serious adverse events. Facial flushing, herpes labialis
Harris 2016	Pilot study	7.4 years	SP PP	6(4/2)	43	(6.5–9)	Autologous BM-MSCs-NPCs	IT	Repeated (2–5)	Four of the six patients showed measureable clinical improvement	No serious adverse events.
Cohen 2017	I	6 months	RR SP	24 (16/8)	46.5	6 (3.0–6.5)	Autologous BM-MSCs	IV	Single	Cell infusion was tolerated well without treatment-related severe or serious adverse events or evidence of disease activation	No serious adverse events

manufacturing practices (GMP)-grade standard protocol for hMSC-derived exosomes have been developed (Pachler et al. 2017).

5 Neural Stem Cell Therapy

5.1 Biology of Neural Stem Cells

Neural stem cells are self-renewing multipotent cells located in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus (Xiao et al. 2017). NSCs can differentiate into both neurons and glia. Either embryonic or adult brain tissue can be used as source of NSCs. They can also be generated from ESCs, MSCs, and iPSCs (Volpe et al. 2016). Due to differentiation capacity of NSCs to OPCs and oligodendrocytes, it is conceivable that NSCs can be used for cell replacement in demyelinating diseases such as MS.

5.2 In Vivo Studies

The therapeutic effect of NSCs in animal models of MS has been shown in several preclinical studies (Table 2). Immunization of SJL/J mice with PLP causes relapsing-remitting-type animal model of MS. But, protein immunization with MOG leads to chronic form of EAE in C57/BL6 mice. In addition to mice, NSC transplantation in EAE was performed in other species including Lewis rat, SD rat, and common marmoset (Ben-Hur et al. 2003; Einstein et al. 2003; Lee et al. 2015; Pluchino et al. 2009). Mostly embryonic or fetal cells have been used as the source of NSCs; however adult NSCs were also transplanted into mice (Pluchino et al. 2005; Wu et al. 2013). NSC transplantation was performed at different time points from the first day of immunization to 35 days after. NSC transplantation at 10 days of post immunization (dpi) delayed disease onset in addition to suppressing clinical findings of EAE (Yang et al. 2009). NSC transplantation in EAE was performed via different routes such as intravenous, subcutaneous, intracerebroventricular, intraspinal, intrathecal, intracerebral, and intranasal. Direct route via intracerebral or intraspinal seems more effective, while peripheral route also suppresses clinical signs of EAE by reducing peripheral immune responses (Einstein et al. 2007). More safe and effective route for administration of NSCs, such as intranasal route, may also be used in MS (Wu et al. 2013).

5.3 Mechanisms of Actions

NSCs exert therapeutic effects by several mechanisms including cell replacement, immunomodulation, trophic support to endogenous repair mechanisms, and stimulation of progenitor cell differentiation (Volpe et al. 2016; Xiao et al. 2017). The cell replacement effect of transplanted NSCs has been reported in limited EAE studies (Ben-Hur et al. 2003). Several studies reported that transplanted NSCs reduce clinical signs and inflammatory findings of EAE even though they persist in perivascular area and they do not migrate toward the lesion site (Ben-Hur et al. 2003; Einstein et al. 2003, 2006). These findings suggest that NSCs exert beneficial effects via other mechanisms such as immune modulation. Local and peripheral immune modulatory effects of these cells were supported by several EAE studies (Einstein et al. 2006, 2003, 2007; Pluchino et al. 2009; Yang et al. 2009). Transplantation of NSCs reduces perivascular infiltrates, CD3+ cells, and ICAM-1 and LFA-1 expression and increases Treg cells in the brain and spinal cord (Einstein et al. 2003, 2006). IL-10 overexpressing adult NSCs significantly suppress CD45+ cells, CD4+ T cells, CD68+ macrophages/microglia, and CD8+ T cells in the spinal cord (Yang et al. 2009). Peripheral immunosuppressive effects of NSCs also contribute to attenuation of clinical findings. NSC transplantation via intravenous route decreased the number of CD3+ T cells and Mac3+ macrophages infiltrating the spinal cord (Einstein et al. 2007). Subcutaneous injection of NSCs inhibits generation of effector T cells, DC maturation, and cytokine production (Pluchino et al. 2009).

Table 2 Neural stem cell transplantation studies in EAE

Organism	Model	Transplanted cells	Genetic modification of cells	Transplantation time	Route of administration	Outcome	References
Lewis rat	SC and CFA	Rat- neonatal striatal spheres		Disease peak	ICV or IT	Cells migrated into the brain or spinal cord (inflamed white matter). Cell differentiation (neuronal and glial)	Ben-Hur et al. (2003)
Lewis rat	SC and CFA	Rat- neonatal striatal spheres		On day immunization	ICV	Decreased clinical severity of EAE and the brain inflammation (reduction in perivascular infiltrates and decreased expression of ICAM-1 and LFA-1)	Einstein et al. (2003)
C57BL/6mice	MOG	NPCs		On day 10,15, and 22 dpi	ICV or IV	Reduction of astrogliosis and marked decrease in the extent of demyelination and axonal loss	Pluchino et al. (2003)
C57B/6 mice	MOG	Neurospheres		On day 6 dpi	ICV	Downregulated inflammatory process, reducing demyelinating process and axonal injury	Einstein et al. (2006)
C57B/6 mice	MOG	hESC-derived NPCs		On day 7 dpi	ICV	Reduction in clinical signs, axonal damage and demyelination, suppression of encephalitogenic T cells	Aharonowiz et al. (2008)
C57BL/6 mice	MOG	ESC-derived NPCs		On the day of immunization or on day 10 dpi	IV	Substantial delay of the disease onset, marked reduction in EAE severity, decreased inflammation and demyelination	Cao et al. (2011)
C57BL/6 mice	MOG	mMSC-NSC		On day 21, 28, and 35 dpi	IT repeated	Reduced immune cell infiltration, area of demyelination, increased number of endogenous nestin+ progenitor cells	Harris et al. (2012)
C57BL/6 mice	MOG	aNSCs		On days 14 and 21 dpi	Intranasal, IV	Reduction in clinical sign, no peripheral immune responses, decreased spinal inflammation	Wu et al. (2013)
C57BL/6 mice	JHMV	hESCs-derived NSC transcriptomic signature-based selection		On day 14 dpi	Intraspinal	Significant neurological recovery, reduced neuroinflammation, decreased demyelination, and enhanced remyelination. Decreased accumulation of CD4+ T cells, increase in Tregs	Chen et al. (2014)
C57BL/6 mice	JHMV	Human EB-derived NSC		On day 14 dpi	Intraspinal	Decreased accumulation of CD4 + Tcells in the CNS, reduced demyelination at the site of injection, transient increase in Tregs	Plaisted et al. (2016)
C57B/6 mice SJL/J mice	MOG, adoptive transfer (PLP)	Mouse neurospheres		On day 8 dpi	IV	Suppression of encephalitogenic T cells, reduced demyelinating process and axonal injury	Einstein et al. (2007)

(continued)

Table 2 (continued)

Organism	Model	Transplanted cells	Genetic modification of cells	Transplantation time	Route of administration	Outcome	References
SJL mice	PLP139–151	Mice SVZ aNPCs		First disease episode or first clinical relapse	IV	Promoted brain repair, induced apoptosis of CNS-infiltrating encephalitogenic T cells	Pluchino et al. (2005)
SJL mice	PLP139–151	Mouse NPCs		On day 3 and 10 dpi or 10 dpi only	SC	Clinical improvement, inhibition of the generation of encephalitogenic T cells, BMP-4-dependent impairment of the DC maturation	Pluchino et al. (2009) b
Common marmoset	MOG 1–125	Human NPCs		Disease onset	IT or IV	Increase of the CCT velocities at the lower limb, decrease in the number of inflammatory infiltrates	Pluchino et al. (2009)a
C57BL/6 mice	MOG	Mouse aNSCs	IL-10-transduced	On day 10, 22, or 30 dpi	IV or ICV	Suppression of clinical sign, enhanced anti-inflammatory effect, induction of T cell apoptosis, promotion of remyelination	Yang et al. (2009)
C57BL/6 mice	MOG, adoptive transfer EAE	Mouse BM-derived NPCs	CCR5-transduced	On day 22 dpi (peak)	IV	Suppressed CNS inflammatory infiltration, myelin damage, and clinical sign of EAE	Yang et al. (2012)
FVB mice, Biozzi ABH mice	MOG	Mouse NPCs	Olig2-transduced	Disease onset or first relapse	ICV	Reduced the clinical signs of acute and relapsing disease	Sher et al. (2012)
C57BL/6 mice	MOG	NSPCs	IL-10 producing	On day 7 dpi or on first sign of disease	ICV, IV	Suppress clinical signs, inhibit T-cell activation, proliferation, and cytokine production	Klose et al. (2013)
SD rats	MOG	Rat/fetal NSCs	IDO	On day 5 dpi	IV	Attenuated clinical scores and faster remission in local immune suppression in the cervical lymph nodes and CNS, reduction in the number of activated T lymphocytes and an increase in Treg	Lee et al. (2015)
C57BL/6 mice	MOG	Mice/BM-derived NSCs	Overexpression IL-10, NT-3, and LINGO-1-Fc	At the onset (day 10 dpi) or chronic stage (day 60 dpi) of disease	IV	Cocktail-NSCs suppress acute and chronic stage of EAE, inducing M2 macrophages/microglia, reducing astrogliosis, and promoting axonal integrity, remyelination, and endogenous oligodendrocyte/neuron differentiation	Li et al. (2016)

Human NSCs (hNSCs) inhibit allogeneic immune cell responses when cocultured with T cells or DCs. They suppress T cell proliferation, decrease DC differentiation from myeloid precursors and maturation, suppress antigen-presenting capacity of DCs, and inhibit costimulatory molecule (CD80, CD86, and MHC-II) expression (Pluchino et al. 2009).

IL-10 overexpressing adult NSCs lead to reduced numbers of CD68+ and CD4+ cells and CD8+ cells, inhibit production of inflammatory cytokines IFNγ and IL-17, and induce apoptosis of encephalitogenic T cells (Yang et al. 2009). Reduced proliferation capacity of autoreactive T cells in draining lymph nodes was observed in EAE mice treated with IL-10-producing NSCs (Klose et al. 2013). Additionally, they inhibit proliferation and cytokine production of T cells. There are some evidence supporting the notion that soluble factors can mediate the immunosuppressive effects of NSCs. NSC supernatant suppresses differentiation of Th17 cells in vitro and in Th17 cell-driven EAE model with IFNγ−/− mice, in vivo (Cao et al. 2011). After testing various cytokines and neurotrophins, leukemia inhibitory factor (LIF) was found as a soluble factor that is responsible from immunosuppressive effects of NSCs (Cao et al. 2011).

5.4 Limitations and Solutions

NSC-based treatment in MS still needs improvement. Transplanted cells usually remain in the perivascular area and do not migrate to the lesion site. To increase the migration capacity, genetically modified NSCs with CCR5 transduction were generated and used in EAE models (Yang et al. 2012). CCR5 transduction accelerated migration of NSCs toward lesion site, even when given intravenously. Several genetic modifications were performed to increase therapeutic effects of NSCs such as Olig2, IL-10, and indoleamine 2,3-dioxygenase (IDO) transduction (Burt et al. 2015; Klose et al. 2013; Nam et al. 2015; Sher et al. 2012; Yang et al. 2009). Finally, cocktail transduction with IL-10, NT-3, and Lingo-1 Fc-engineered cells was used to enhance immunosuppressive and cell protective effect of NSCs (Zhang et al. 2016).

Another trouble in NSC therapy in MS is inflammatory microenvironment at lesion sites during demyelination. Several soluble factors and microglial cells affect transplanted NSCs' viability, differentiation, and migration capacity to the lesion site. Nonpermissive microenvironment conditions can be reversed by different modulations of NSCs. Preconditioning of NSCs with minocycline enhances survival of grafted cells, increases proliferation of NSCs, and induces release of cytoprotective factors such as Nrf2 (Sakata et al. 2012). Treatment of NSCs with IL-10 and IL-4 increases expression of adhesion molecules LFA-1 and chemokine receptors CXCR4 and CCR5 and enhances their migration capacity (Guan et al. 2008).

Due to the differentiation capacity to all neuronal cell lineages and the presence of anti-inflammatory and trophic effects, NSCs are ready for clinical applications. A phase 1 NSC transplantation study was performed in patients with Pelizaeus–Merzbacher disease (PMD), which is a leukodystrophy characterized by hypomyelination (Gupta et al. 2012). Results demonstrated that allogeneic NSCs transplantation is safe and effective in PMD. Transplanted cells engrafted to recipient tissue and produced myelin.

6 Induced Pluripotent Stem Cells

6.1 Generation of Induced Pluripotent Stem Cell

Induced pluripotent stem cells (iPSCs) are pluripotent stem cells produced from adult somatic cells by reprogramming process using particular transcription factors. They were first generated from mouse embryonic and adult fibroblasts by Shinya Yamanaka and his colleagues at Kyoto University. Yamanaka's group used retroviral vectors Octamer 3/4 (OCT3/4), SRY-box-containing gene 2 (SOX2), cytoplasmic Myc protein (c-MYC), and Krueppel-like factor 4 (KLF4) for reprogramming both mouse and human

fibroblasts, whereas the group of James Thomson used lentiviral vectors encoding OCT4, SOX2, NANOG, and Lin28 for reprogramming human fibroblasts. These iPSCs exhibited similar characteristics with ESCs as they can self-renew and differentiate into all cell types in the body. Several factors affect reprogramming efficiency including initial cell type, reprogramming factors, delivery method, and culture conditions. Apart from fibroblasts, several types of somatic cells have been used for iPSC generation, but fibroblast is still the favorable cell type for iPSC generation. Classical reprogramming factors have tumor-forming capacities. Therefore, new efficient reprogramming methods by using chemicals and microRNAs were developed. Retroviral and lentiviral vectors have been mainly used to deliver these factors into somatic cells. The major disadvantage of the original delivery method is that viral vectors integrate into genome of iPSCs and may cause tumor development. New approaches such as the use of nonviral delivery methods or omitting the oncogenic factors c-MYC and KLF4 could prevent tumorigenicity (Durnaoglu et al. 2011; Harding and Mirochnitchenko 2014).

6.2 In Vivo Studies

The recent successful improvements in generation of iPSCs and their differentiation into neural precursor cells (NPCs) and oligodendrocyte precursor cells (OPCs) initiated autologous iPSC therapy studies in MS. First, in vivo therapeutic effect of iPSCs was evaluated in EAE, chemically induced demyelination, and genetic hypomyelination models (Table 3). Mouse iPSC-derived NPCs (miPSC-NPCs) were transplanted to C57Bl/6 mice with MOG-induced EAE intrathecally after disease onset (Laterza et al. 2013). miPSC-NPC treatment reduced clinical scores of EAE and decreased demyelinated areas and axonal damage in the spinal cord. Transplanted miPSC-NPCs did not differentiate to neither neuron nor oligodendrocyte and did not migrate from perivascular space to lesion site. Neuroprotective effects of miPSC-NPCs were partly through the secretion of LIF that support resident oligodendrocyte survival and differentiation. Microarray analysis revealed that miPSC-NPCs counterbalanced the EAE-associated transcriptional changes in the spinal cord. These cells also limit BBB damage and decrease CNS-infiltrating inflammatory cells (Laterza et al. 2013). Intraventricular transplantation of miPSCs also improved the functional recovery of EAE mice and reduced T cell infiltration and white matter damage (Zhang et al. 2016). The effect of hiPSC-derived embryoid body intermediate-stage NPCs (EB-NPCs) was examined in neurotropic JHM strain of mouse hepatitis virus (JHMV)-induced EAE (Plaisted et al. 2016). Significant clinical recovery was not observed in EB-NPCs transplanted mice. EB-NPCs were rapidly eliminated, but they decreased accumulation of CD4+ T cells in the CNS, reduced demyelination at the site of injection, and increased the number of Treg cells (Plaisted et al. 2016). While healthy hiPSC-derived NPCs decreased inflammation, PPMS patient-derived NPCs failed to provide any profit in demyelination process (Nicaise et al. 2017).

OPCs derived from iPSCs were also transplanted into animal models of MS. Intracerebrally transplanted OPCs derived from miPSCs and hiPSCs survive and differentiate to MBP-expressing oligodendrocytes in both cuprizone- and lysolecithin-induced models (Czepiel et al. 2011; Nicaise et al. 2017). The recovery effect of OPC transplantation on demyelination was also confirmed in congenital hypomyelination model (Douvaras et al. 2014; Terzic et al. 2016; Wang et al. 2013). Transplanted OPCs differentiate into MBP-expressing oligodendrocytes (Terzic et al. 2016) and contribute to myelination (Douvaras et al. 2014; Terzic et al. 2016; Wang et al. 2013). The effect of iPSC-derived OPC transplantation was also evaluated in EAE model (Thiruvalluvan et al. 2016). Transplanted OPCs reduced EAE scores, cell infiltration, and demyelination in the cerebellum. Histological analysis revealed that transplanted OPCs remained within the ventricles; therefore their effect on clinical and histological features of EAE occurs most

Table 3 Induced pluripotent stem cells derived cell therapies in demyelinated animal models

Organism	Model	Original cell	Intermediate cell	Transplanted cell	Route of administration	Outcome	References
C57Bl/6 mice	Cuprizone induced	Mouse embryonic fibroblast	miPSCs	OPCs	IC (corpus callosum)	Differentiate to MBP-expressing oligodendrocytes, contributed to the remyelination	Czepiel et al. (2011)
C57Bl/6 mice	Cuprizone induced	Human blood cells with PPMS	hiPSCs	NPCs	IV	NPCs from PPMS patients failed to provide any benefit in preserving compact CNS myelination during active demyelination	Nicaise et al. (2017)
Sprague Dawley rats	Lysolecithininduced	Human fibroblast	hiPSCs	OPCs	IC (optic chiasm)	Recovery from symptoms (measured with visual evoked potential), transplanted cells survived and integrated within the chiasm, differentiated to PLP and/or MBP expressing oligodendrocytes, contributed to the remyelination	Pouya et al. (2011)
C57Bl/6 mice	MOG 35–55 induced EAE	Mouse embryonic fibroblasts	miPSCs	NPCs	IT	Decreased EAE score, reduction of demyelinated areas and axonal damage, transplanted miPSC-NPCs counterbalanced the EAE-associated transcriptional changes	Laterza et al. (2013)
C57BL/6 mice	MOG 35–55 induced EAE	miPSCs	miPSCs	NPCs	Intraventricular	Reduced T cell infiltration, ameliorated WM damage	Zhang et al. (2016)
C57BL/6 mice/ marmoset	Human MOG 34–56 induced EAE	Human fibroblast	hiPSCs	OPCs	ICV (cisterna magna), IC	Significant reduction of subsequent EAE scores and cell infiltration; transplanted hiPSCs in ventricule, migration and differentiation to MBP producing cells in marmoset model	Thiruvalluvan et al. (2016)
C57BL/6 mice	Intracranially JHMV	Primary fetal human fibroblasts	hiPSCs	NPCs	Intraspinal	EB-NPCs were rapidly rejected, decreased accumulation of CD4+ T cells in the CNS, reduced demyelination at the site of injection, modest pathological improvements, no significant clinical recovery	Plaisted et al. (2016)
Shiverer mice	Congenital hypomyelination	Mouse embryonic fibroblast	miPSCs	OPCs	IC (corpus callosum and striatum)	Transplanted cells expressing MBP	Terzic et al. (2016)
Shiverer mice	Congenital hypomyelination	Human fibroblast, keratinocyte	hiPSCs	OPCs	IC (5-site forebrain and brainstem)	Increased the survival, myelination of the brain, brainstem, and cerebellum	Wang et al. (2013)
Shiverer mice	Congenital hypomyelination	Human fibroblast with PPMS	hiPSCs	OPCs	IC (forebrain)	Host mouse axons were ensheathed, mature compact myelin observed with electron microscope	Douvaras et al. (2014)

(continued)

Table 3 (continued)

Organism	Model	Original cell	Intermediate cell	Transplanted cell	Route of administration	Outcome	References
Shiverer mice	Congenital hypomyelination	Mouse embryonic fibroblast	iNSCs	iNSCs	IC	MPB+ myelin sheets were detected in white matter tracts of the cerebellum	Lujan et al. (2012)
Shiverer mice	Congenital hypomyelination	Mouse embryonic fibroblast	iOPCs	iOPCs	The dorsal region of the spinal cord	Transplanted cells colonized the dorsal column white matter, generated compact myelin sheaths around dorsal column axons	Najm et al. (2013)
Shiverer mice	Congenital hypomyelination	Rat fibroblast	iOPCs	iOPCs	IC (corpus callosum and cerebellum)	Oligodendroglial ensheathment of host axons by the transplanted iOPCs, myelin formation	Yang et al. (2013)

likely through secreted factors. In contrast to this, intracerebrally transplanted hiPSC-derived OPCs migrated toward the lesion and differentiated to MBP producing mature oligodendrocytes in marmoset model of EAE, suggesting that differences between species or route of administration are important (Thiruvalluvan et al. 2016). Genetic modification of transplanted cells can be used to enhance their targeted migration. Polysialylating enzyme sialyltransferase X (STX) overexpression in iPSC-derived OPCs increased their migration along the axons in cuprizone-induced model (Czepiel et al. 2014).

After the successful reprogramming of somatic cells to iPSCs, more direct neural lineage conversion methods have been developed. Functional neurons were obtained by transdifferentiation of fibroblasts using defined factors. Afterward, similar methods were developed for the generation of iNSCs and iOPCs using neural lineage-specific sets of TFs. iNSC and iOPC transplantations were performed in dysmyelinated *Shiverer* mice (Lujan et al. 2012; Najm et al. 2013; Yang et al. 2013). Transplanted iNSCs differentiated into oligodendrocytes capable of integration into dysmyelinated *Shiverer* brain (Lujan et al. 2012). iOPCs when given into the spinal cord, corpus callosum, and cerebellum survived, ensheathed host axons, and produced myelin (Najm et al. 2013; Yang et al. 2013).

6.3 Mechanisms

Because of inadequate endogenous remyelination, cellular therapy is moving forward in MS treatment. Therapeutic effects of iPSCs are not limited with cell replacement. iPSCs also exhibit immunosuppressive effect and provide trophic support on endogenous repair mechanisms. NPC transplantation decreases T cell infiltration (Plaisted et al. 2016; Zhang et al. 2016). Secreted LIF from transplanted NPCs exerts trophic action on endogenous oligodendrocytes (Laterza et al. 2013).

Apart from cell replacement, iPSCs have been used in vitro to model diseases in order to understand the underlying mechanisms and for screening drugs that modify the disease process. MS patient-specific iPSCs were first generated in 2011 by using fibroblasts from a 35-year-old patient with RRMS (Song et al. 2012). Patient-derived iPSCs were successfully differentiated to neural progenitors and mature neurons. Subsequently, patient iPSCs were also generated from PPMS patients (Douvaras et al. 2014; Nicaise et al. 2017). In the study by Douvaras et al., four iPSC lines were converted to NSCs and OPCs which carry normal karyotypes. Transplanted OPCs provided myelination in *Shiverer* mice. Similar studies should be continued especially in MS patients with high genetic load for disease modeling.

6.4 Practical Considerations

There are still some concerns about iPSC-based treatment in MS. Firstly, iPSC generation methods still need to be improved. The other concern is preference of initial cell type for transplantation. OPCs are superior for cell replacement, but anti-inflammatory and trophic effects of these cells have not been demonstrated yet. NSCs can differentiate into all neuronal cell lineages and also have anti-inflammatory and trophic effects, but they may differentiate to astrocytes as well and therefore lead to unwanted astrogliosis in MS. Also, they have tumorigenicity potential. Another major concern is the route of administration of cells. Direct intracerebral or intraspinal injection seems more effective, but they are not practical in clinical setting. Intranasal route may also be used effectively, and it is a less invasive route for administration of iPSCs in MS (Wu et al. 2013). Finally, use of allogeneic or autologous iPSCs should be considered. Autologous iPSCs are preferable, but they may be ineffective due to intrinsic disease factors (Nicaise et al. 2017). To avoid immunogenicity, healthy

allogeneic iPSC therapy needs immunosuppressive treatment that may cause MS relapses.

7 Challenges and Future Perspectives of Stem Cell Transplantation for MS

Stem cell transplantation can be regarded as a potential source of treatment for MS. Nevertheless, before introducing stem cell treatment wholesale into clinics, methodological, ethical, and clinical challenges must be overcome in stem cell therapy studies.

7.1 Sources

HSCs and MSCs have been used in clinical MS stem cell trials. In spite of the better availability of HSCs and MSCs compared to NSCs, remyelination capacity of NSCs makes them the preferred cell type for MS stem cell clinical trials particularly in the progressive stages of MS (Mariano et al. 2015; Sarkar et al. 2017). Although iPSC-derived stem cells have not been used in clinical trials of MS, they are suitable candidates for individualized cell replacement therapy due to their advantage of being easily obtained from the patient's own tissue.

The autologous and allogeneic stem cells contain different advantages and disadvantages in MS treatment (Cohen 2013). Autologous stem cell is less immunogenic, but generation from PSCs takes a longer time period, which makes it disadvantageous particularly in the acute phase of MS because of the necessity of immediate SCT. Additionally, autologous stem cells may be ineffective due to intrinsic disease factors (Nicaise et al. 2017). However, genetic defects can be corrected with several gene-editing methods including zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems (Maeder and Gersbach 2016), which means that autologous stem cells could be used in MS therapy upon genome editing, if necessary. The use of allogeneic stem cells has advantages such as avoiding the risk by genetic susceptibility of the recipient to develop MS. Since these cells are readily available from biobanks (Natalwala and Kunath 2017), they may be useful in the acute and progressive phase of MS.

7.2 In Vitro Cell Expansion and Manipulation

Numerous technical factors affect the yield, viability, function, and efficacy of SCT (Bang et al. 2016). The use of fetal bovine serum in culture medium raises safety concerns, including possible transmission of zoonoses and infusion-related allergic reactions (Cohen 2013). Stem cell production for clinical trials should be done under current GMP standards (Galvez-Martin et al. 2016).

Various approaches for in vitro cell expansion increase the stem cell proliferation, survival, and trophic support and reduce senescence of stem cells (Bang et al. 2016). Preclinical studies showed that ex vivo treatment of stem cells with trophic factors or chemical agents enhanced the migration of stem cells and trophic support in the brain. Lastly, genetic modification of stem cells, such as overexpressing chemokine receptors or IL-10, increased their efficacies and migration capacities in animal models of MS (Klose et al. 2013; Phillips and Tang 2008; Yang et al. 2012).

7.3 Practical Considerations

7.3.1 Storage

The ability to freeze with xeno-free freezing media and storing them in proper conditions are essential steps for clinical use of stem cells (Sarkar et al. 2017). Automated methods of thawing cells may increase viability of cells (Nishiyama et al. 2016). Other considerations

for storage conditions are cell number, cell density, vial size, and total volume.

7.3.2 Passage Number

The passage number of transplanted stem cells affects their proliferation, differentiation, and therapeutic capacities (Sisakhtnezhad et al. 2017). For example, lower than five passages of MSCs appear to be optimal for transplantation (Liu et al. 2016).

7.3.3 Route

The routes of cell administration vary among studies. Direct intracerebroventricular or intrathecal injections seem more effective, but IV route could be superior for peripheral immunosuppressive effect (Cohen 2013). However, entry to CNS is very low, and most of the injected cells are trapped by the lung, liver, spleen, and lymph nodes. Effective and less invasive routes for administration of stem cells such as intranasal route may also be used in MS (Li et al. 2015).

7.3.4 Dose

As the differences in the number of doses could be an important factor to the observed variation in responses, standardization is required for optimal doses for each transplanted cell type (Cohen 2013). Additionally, repeated administrations should be used to increase clinical benefit especially in progressive MS (Harris et al. 2016).

7.4 Tracking of Transplanted Cells

Noninvasive cell-tracking methods allow real-time monitoring of survival, migration, and homing of administered stem cells. MRI, magnetic particle imaging, positron emission tomography, single-photon emission computed tomography, and optical imaging methods can be used for in vivo tracking of stem cells (Filippi et al. 2017a; Ngen and Artemov 2017). To date, an optimal technique for in vivo cell tracking does not yet exist in the clinical setting.

7.5 Risks

In general, HSCT and MSC transplantation in MS patients have been well-tolerated, but several potential acute and chronic adverse effects should be considered. Infusion-related toxicity and infection are acute risks of stem cell therapy, and additive immunosuppressive treatment increases the infection risk following transplantation (Sarkar et al. 2017). Ectopic tissue formation and malignant transformation of stem cells are theoretical concern, although it has not been reported in MSC therapy studies (Cohen 2013). iPSCs have more tumorigenicity potential depending on pluripotency induction method. The use of iOPCs or iNSCs obtained by direct reprogramming without pluripotent stage can avoid tumor development risk (Xie et al. 2016). The other main risk in stem cell treatment is allogeneic immune rejection that is primarily mediated by T cell-dependent immune responses and needs lifelong immunosuppressive treatment. Previous studies supported that SCTs may also lead to disease activation in MS, presumably by leading to fever (Cohen 2013).

7.6 Cost-Effectiveness

The initial costs of stem cell therapies are extremely high, and cost-effective stem cell treatments must be developed (Sarkar et al. 2017). To assess the risk-benefit ratio of stem cell therapies, randomized studies should be performed comparing the efficacy of stem cells against other conventional therapies.

Stem cell tourism is a ridiculous term used to describe traveling abroad to undergo medical stem cell treatments that are not approved or available in patients' home country. Anecdotal evidence suggests that stem cell tourism leads to physical and financial risk to patients (Marks et al. 2017). Clinicians and regulators should work together to prevent deregulated cell-based therapies.

8 Conclusion

Stem cell-based therapies are attractive alternatives for the treatment of MS. There are still some issues which need to be resolved such as low efficacy of iPSCs, lack of proper differentiation protocols, epigenetic alternation in donor cells, and heterogeneity of transplanted cells. Improvement of stem cell technology will contribute to overcome these problems. For example, screening and selection of viable, genetically stable, and desired stem cells can prevent tumorigenicity and immunogenicity side effects of SCT.

Apart from these technical problems, several clinical difficulties should be considered. One of main concerns in SCT is to make a choice between autologous versus allogeneic stem cells for MS treatment. In older MS patients, HLA-matched young donor cells for transplantation may be more appropriate (Phanthong et al. 2013).

Combination therapy with anti-inflammatory and remyelinating agents (Anti-Lingo1 ab) and also other stem cells should be considered to increase the efficacy of stem cell therapy (Harlow et al. 2015). Emerging, innovative treatment approaches such as using cell-free stem cell products (conditioned media, exosome) should be investigated in animal models of MS (Derkus et al. 2017; Harlow et al. 2015).

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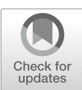

Stem Cells from Human Extracted Deciduous Teeth Expanded in Foetal Bovine and Human Sera Express Different Paracrine Factors After Exposure to Freshly Prepared Human Serum

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Abstract

Background: The response of stem cells to paracrine factors within the host's body plays an important role in the regeneration process after transplantation. The aim of this study was to determine the viability and paracrine factor profile of stem cells from human extracted deciduous teeth (SHED) pre-cultivated in media supplemented with either foetal bovine serum (FBS) or pooled human serum (pHS) in the presence of individual human sera (iHS).

Methods: SHED ($n = 3$) from passage 4 were expanded in FBS (FBS-SHED) or pHS (pHS-SHED) supplemented media until passage 7. During expansion, the proliferation of SHED was determined. Cells at passage 7 were further expanded in human serum from four individual donors (iHS) for 120 h followed by assessment of cell viability and profiling of the secreted paracrine factors.

Results: Proliferation of SHED was significantly higher ($p < 0.05$) in pHS supplemented media compared to FBS supplemented media. pHS-SHED also maintained their higher proliferation rate compared to FBS-SHED in the presence of iHS. In iHS supplemented media, FBS-SHED expressed significantly higher levels of SDF-1A ($p < 0.05$) after 24 h compared to pHS-SHED. Similar results were found for HGF ($p < 0.01$), LIF ($p < 0.05$), PDGF-BB ($p < 0.05$), SDF-1A ($p < 0.01$), and IL-10 ($p < 0.05$) when cell culture supernatants from FBS-SHED were profiled 120 h post-incubation.

Conclusion: SHED expanded in pHS instead of FBS have higher proliferative capacity and show an altered secretion profile. Further studies are needed to determine whether these differences could result in better engraftment and regeneration following transplantation.

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Keywords
Mesenchymal stem cells · Paracrine factors · Proliferation · Regenerative medicine · SDF-1

Abbreviations

CPD	cumulative population doubling time
FBS	foetal bovine serum
FGF-2	fibroblast growth factor 2
G-CSF	granulocyte colony stimulating factor
HGF	hepatocyte growth factor
IGF-1	insulin like growth factor 1
iHS	individual human sera
IL	interleukin
ISCT	International Society for Cellular Therapy
LIF	leukaemia inhibitory factor
MSCs	mesenchymal stem cells
PDGF-BB	platelet-derived growth factor BB
pHS	pooled human serum
SCF	stem cell factor
SDF-1A	stromal cell-derived factor-1A
SHED	stem cells from human extracted deciduous teeth
VEGF	vascular endothelial growth factor

1 Introduction

In the last few decades, cell-based regenerative therapies have received considerable attention in the field. Multipotent mesenchymal stem cells (MSCs) are considered one of the best sources of stem cells for regenerative therapy. Several in vitro and in vivo studies have shown promising regenerative outcomes after transplantation of MSCs in different pathological scenarios (Gnanasegaran et al. 2017; Williams et al. 2013; Miyahara et al. 2006). However, most of the clinical trials using MSCs reported only short-term regenerative benefits that were linked to their low retention following transplantation (Haque et al. 2015; Trounson and McDonald 2015).

MSCs reside in the perivascular region of almost all tissues and organs of the human body (Kalinina et al. 2011; da Silva Meirelles et al. 2006). The number of MSCs within the tissues is relatively low; hence in vitro expansion prior to transplantation is needed to acquire therapeutically relevant cell numbers (Haque and Abu Kasim 2017). MSCs are often expanded in media supplemented with foetal bovine serum (FBS) that contains xenoantigens and could potentially impair the regenerative potential of MSCs (Haque et al. 2015). Recently, human platelet lysates, human cord blood serum, and allogenic pooled human serum (pHS) have been used as media supplements for MSC expansion. Moreover, it has been suggested that these supplements are suitable for in vitro expansion of MSCs (Haque and Abu Kasim 2017; Bieback et al. 2012; Blazquez-Prunera et al. 2017b). Several recent studies suggested that the immunomodulatory properties of MSCs are dependent on the type of supplements such as FBS, pHS, and commercially produced cell culture supplement used for their expansion (Blazquez-Prunera et al. 2017a; Komoda et al. 2010). However, no major complications have been reported in clinical trials using MSCs expanded with different supplements (Yubo et al. 2017; Yim et al. 2016). Thus, we hypothesized that xeno-contamination might not be the only factor affecting engraftment and regenerative outcomes after MSC transplantation. Notably, regeneration represents a highly complex process and involves a large number of factors including paracrine signalling molecules, extracellular vesicles, and the extracellular matrix (Vunjak-Novakovic and Scadden 2011; Wagers 2012; Bassat et al. 2017). Moreover, endogenous cytokine gradient have been reported to play a vital role in the directional migration and engraftment of transplanted MSCs (Youn et al. 2011; Park et al. 2017). Hence, following transplantation, appropriate response of the transplanted cells to the host's microenvironment is important for successful engraftment of the cells and the subsequent regeneration of the targeted organ or tissue. Furthermore, secretion of paracrine factors by the graft cells could negatively impact the host's cytokine homeostasis.

SHED are neural crest-derived ectomesenchymal stem cells that, if cultivated in presence of FBS, exhibit all crucial properties of MSCs including the expression pattern, differentiation capacity, and immunotolerance after transplantation (Kaltschmidt et al. 2012; Sloan and Waddington 2009). In addition, it has been suggested that SHED are well tolerable and might have a proliferation and differentiation potential superior to MSCs from other sources (Wang et al. 2012; Nakamura et al. 2009).

As the paracrine factors play a vital role in the processes of proliferation, migration, and homing of transplanted stem cells including SHED, we analyse the cell viability and expression of paracrine factors in SHED expanded in FBS and pHS supplemented media that have been exposed to freshly prepared human serum in an attempt to simulate the graft microenvironment. In this study we report that SHED expanded in the FBS have lower proliferative capacity and secrete higher level of paracrine factors in the presence of iHS compared to SHED expanded in pHS.

2 Materials and Methods

2.1 Ethics Approval

All the samples were obtained following an informed written consent. Sample collection procedures were approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference #DF RD1301/0012[L] for blood collection; DFCD0907/0042[L] for teeth collection). An overview of the experimental strategy study is shown in Fig. 1.

2.2 Isolation and Expansion of SHED

SHED were isolated as described earlier (Govindasamy et al. 2010; Haque and Abu Kasim 2017).

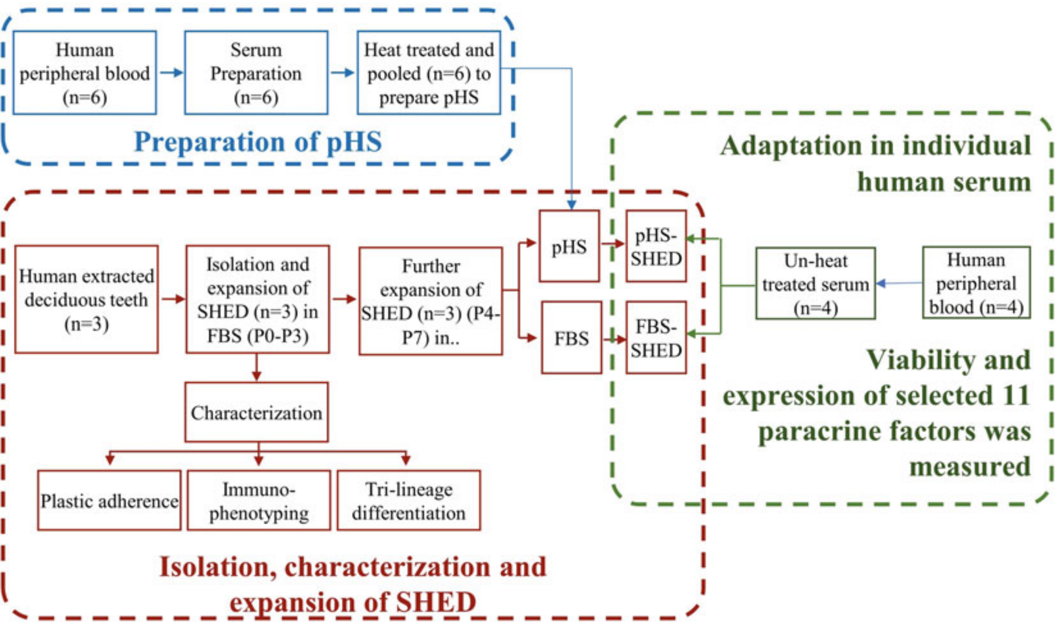

Fig. 1 Schematic overview of the experimental strategy. (*SHED* stem cells from human extracted deciduous teeth, *FBS* foetal bovine serum, *pHS* pooled human serum, *P* passage, *FBS-SHED* SHED expanded in FBS supplemented medium, *pHS-SHED* SHED expanded in pHS supplemented medium)

2.3 Assessment of MSC Characteristics in SHED

According to the guidelines by the International Society for Cellular Therapy (ISCT), MSCs should fulfil the following criteria: they must adhere to plastic, express specific surface markers, and be capable of tri-lineage differentiation in vitro (Dominici et al. 2006).

Plastic adherence of SHED was confirmed by using an inverted microscope (Primo Vert, Carl Zeiss, Germany). Expression of specific surface antigens on SHED and tri-lineage differentiation potential were determined as described earlier (Haque and Abu Kasim 2017).

2.4 Preparation of Human Serum

Blood was collected from healthy male donors aged 21–35 years. Exclusion criteria were as follows: smoking, alcohol consumption, drug and/narcotics addiction, chronic diseases and diseases diagnosed within 4 weeks prior to the blood collection, major surgical treatment in the last 24 months, and immunotherapy. Blood was collected by trained health nurses at the Oro-Maxillofacial Surgical and Medical Sciences Department, Faculty of Dentistry, University of Malaya. Serum was prepared as described before (Haque and Abu Kasim 2017).

Briefly, 20 ml of blood was collected from each donor ($n = 6$), transferred into a 50 ml sterile centrifuge tube (Falcon®, Corning, NY, USA) containing no anticoagulant and allowed to stand at room temperature for an hour to facilitate coagulation. The coagulated blood was centrifuged at $400 \times g$ for 15 min. After subsequent centrifugation at $1800 \times g$ for 15 min, the final serum supernatant was incubated at 57 ± 2 °C for 30 min to obtain a complement inactivated human serum. Six heat-inactivated sera ($n = 6$) were combined to prepare the pHS.

iHS from four donors was prepared according to the procedure mentioned above with the minor modification. In particular, after the final round of centrifugation, the serum supernatant was left untreated to retain complement activity. pHS and non-inactivated iHS were sterilized by filtration through a 0.2 μm membrane filter (Nalgene™, Thermo Fisher Scientific, NY, USA).

2.5 Cultivation of SHED

SHED ($n = 3$) were maintained in 10% FBS supplemented Knockout™-DMEM (Gibco®, Thermo Fisher Scientific) until passage 3. Subsequently (from passages 4–7), cultures were maintained in Knockout™-DMEM supplemented with either 10% FBS (Gibco®, Thermo Fisher Scientific, Lot No. 10270) or 10% pHS. Animal-derived component-free TrypLE™ Express (Gibco®, Thermo Fisher Scientific) was used as cell dissociation reagent.

2.6 Effects of FBS and pHS on Proliferation of SHED

Cells from passage 5 to passage 7 were counted by using Trypan Blue (Gibco®, Thermo Fisher Scientific) dye exclusion method. Three technical replications for each biological samples ($n = 3$) were performed, and the average number of cells were used to determine the population doubling time (PD) of SHED from each donor. Data were analysed and plotted using Microsoft Excel. PD at each passage was calculated by using the following equation:

$$X = \frac{\log_{10}(N_H) - \log_{10}(N_I)}{\log_{10}(2)}$$

where X = population doublings, N_I = inoculum number, and N_H = cell harvest number. To obtain the cumulative population doubling time (CPD), the PD increase at the given passage was added to the PD of previous passages (Cristofalo et al. 1998; Li et al. 2015).

2.7 Viability of FBS-SHED and pHS-SHED in Individual Human Serum

SHED maintained in FBS and pHS supplemented medium (FBS-SHED and pHS-SHED, respectively) were seeded (passage 7) at a density of 100,000, 50,000, 25,000, 12,500, and 6255 cells/well in 96-well plates containing Knockout™--DMEM supplemented with 10% iHS (n = 4). After 24 h of incubation at 37 °C in 95% humidified air and 5% CO_2, the viability of SHED was analysed using PrestoBlue™ Cell Viability Reagent (Invitrogen™, Thermo Fisher Scientific). In brief, all the media were discarded after 24 h of incubation, and the wells were washed twice with DPBS. Knockout™-DMEM with 10% PrestoBlue™ reagent (v/v) was added to each well, and the plates were further incubated for 2 h. Absorbance was measured at 570 nm with reference wavelength set to 600 nm using a microplate reader (Infinite 200 PRO, Tecan, Switzerland). The absorbance values were converted to the corrected absorbance of PrestoBlue™ reagent.

2.8 Immunoassay

At passage 7, FBS-SHED and pHS-SHED were seeded in Knockout™-DMEM supplemented 10% iHS, and supernatants were collected after 24 and 120 h post-incubation. Supernatants were used to measure the amount of selected paracrine factors by using Luminex-based ProcartaPlex human cytokine/chemokine 11plex immunoassay kit (Affymetrix, e-Bioscience, Vienna, Austria). The analysed paracrine factors were selected based on their involvement in cell survival and regulation of regeneration (Table 1).

2.9 Data Analysis

Data were analysed using independent sample t-test (SPSS version 22), and $p < 0.05$ was considered significant.

3 Results

3.1 SHED Exhibit MSC Characteristics

Morphological analysis using an inverted microscope revealed a homogenous monolayer soft plastic adherent cells, typical for MSCs (Fig. 2a, b). Using flow cytometry, we validated the expression of MSC positive markers (CD73, CD90, CD105) on the 95% SHED. Moreover, absence of CD14, CD20, CD34, and CD45 in majority of SHED was confirmed (Fig. 2c). The tri-lineage differentiation potential (adipogenic, chondrogenic, and osteogenic) of SHED was confirmed following directed differentiation (Fig. 2d–f). Notably, osteogenic, adipogenic, and chondrogenic cells were detected only in cells subjected to differentiation conditions, while no differentiation was seen in control culture. These properties confirm that the isolated cells were SHED that have the MSCs like properties.

3.2 SHED Expanded in pHS Maintain a Highly Proliferative Phenotype in iHS

Significantly higher proliferation of SHED was observed in pHS compared to FBS supplemented media (Fig. 3a, b). SHED seeded at density of 25000cells/well or lower showed significantly higher viability in pHS supplemented medium after 24 h of incubation (Fig. 3c). In the presence of iHS, significantly higher viability of pHS-SHED was also observed (Fig. 3d).

3.3 SHED Expanded in FBS Express Higher Levels of Paracrine Factors in iHS

After 24 h of incubation in the presence of iHS, significantly higher expression of SDF-1a was detected in FBS-SHED cell culture supernatants compared to that in pHS-SHED. The expression levels of FGF-2, HGF, LIF, PDGF-BB, VEGF,

Table 1 Selected paracrine factors analysed in the current research

Name of the paracrine factor	Function (references)
Fibroblast growth factor 2 (FGF-2)	Shows mitogenic effect (Salcedo et al. 1999; Werner and Grose 2003); enhances proliferation of human BM-MSCs by activation of JNK signalling (Ahn et al. 2009); slows down telomere shortening and the ageing process of MSCs (Yanada et al. 2006; Bianchi et al. 2003); provides cytoprotection (Werner and Grose 2003); induces CXCR4 expression on cells and helps angiogenesis (Salcedo et al. 1999)
Granulocyte colony stimulating factor (G-CSF)	Enhances cellular proliferation, migration, and chemotactic properties (Murakami et al. 2013); prevents apoptosis (Murakami et al. 2013); induces HSC and MSC mobilization from the bone marrow (Kawada et al. 2004)
Hepatocyte growth factor (HGF)	Induces proliferation, survival, migration, and site-specific homing of various cell types including MSCs (Forte et al. 2006; Son et al. 2006)
Leukaemia inhibitory factor (LIF)	Supports self-renewal and maintains multi-differentiation potential of MSCs and other stem or progenitor cells (Metcalf 2003; Kolf et al. 2007)
Platelet derived growth factor BB (PDGF-BB)	Enhances expansion, migration, and survival of MSCs (Fierro et al. 2007; Tamama et al. 2006; Krausgrill et al. 2009)
Stem cell factor (SCF), KIT ligand	Regulates proliferation, differentiation, migration, and homing of several cell types including HSCs and MSCs (Lennartsson and Ronnstrand 2012; Pan et al. 2013)
Stromal cell-derived factor-1a (SDF-1A)	Enhances site-specific migration and homing of MSCs by regulating SDF1/CXCR4 pathway (He et al. 2010; Yu et al. 2015)
Vascular endothelial growth factor A (VEGF-A)	Enhances proliferation and survival of MSCs (Pons et al. 2008); promotes angiogenesis, anti-apoptotic, and immunomodulatory properties (Sulpice et al. 2009; Wang et al. 2006)
Interleukin 4 (IL-4)	Induces activation of B cells; stimulates proliferation of T cells; regulates differentiation of T lymphocytes to T helper cell 2 (T_{H2}) (Choi and Reiser 1998)
Interleukin 6 (IL-6)	Stimulates acute-phase proteins production (Fattori et al. 1994); induces chronic inflammatory responses by stimulating T and B lymphocytes (Gabay 2006)
Interleukin 10 (IL-10)	Promotes immunosuppressive functions by inhibiting activities of T_{H1} cells, natural killer cells, and macrophages (Couper et al. 2008; Pierson and Liston 2010); induces antibody production from activated B cells and amplifies humoral responses (Rousset et al. 1992)

and IL-6 were higher in FBS-SHED supernatants. In contrast, marginally higher expression levels of SCF, G-CSF, and IL-10 were observed in the cell culture supernatants of pHS-SHED (Fig. 4).

After 120 h of incubation, significantly higher expression levels of HGF, LIF, PDGF-BB, SDF-1, and IL-10 were detected in the FBS-SHED supernatants. Moreover, we detected higher expression of FGF-2 and IL-6. Higher expression of SCF, VEGF-A, and IL-4 was detected in pHS-SHED supernatants (Fig. 4).

4 Discussion

Pulp tissue extracted from a deciduous tooth is very small in volume and not suitable to attempt to isolate and expand them in different media. Moreover, isolation of dental pulp-derived stem cells (DPSC) was not successful at lower human serum concentration (20%<) (Khanna-Jain et al. 2012). The concern regarding xeno-contamination due to isolation and expansion of SHED in xenogeneic serum supplemented media can be minimized by culturing them in human serum supplemented media for 2 weeks only (Komoda et al. 2010). Hence, in this study SHED were isolated and expanded in FBS supplemented medium until passage 3 to get enough cells to use them for different experimental purposes.

Prior to conduct researches using MSCs from any sources, their minimum criteria set by the ISCT needed to be studied (Dominici et al. 2006). Usually, the differentiation potential and phenotypic marker expression on MSCs are not

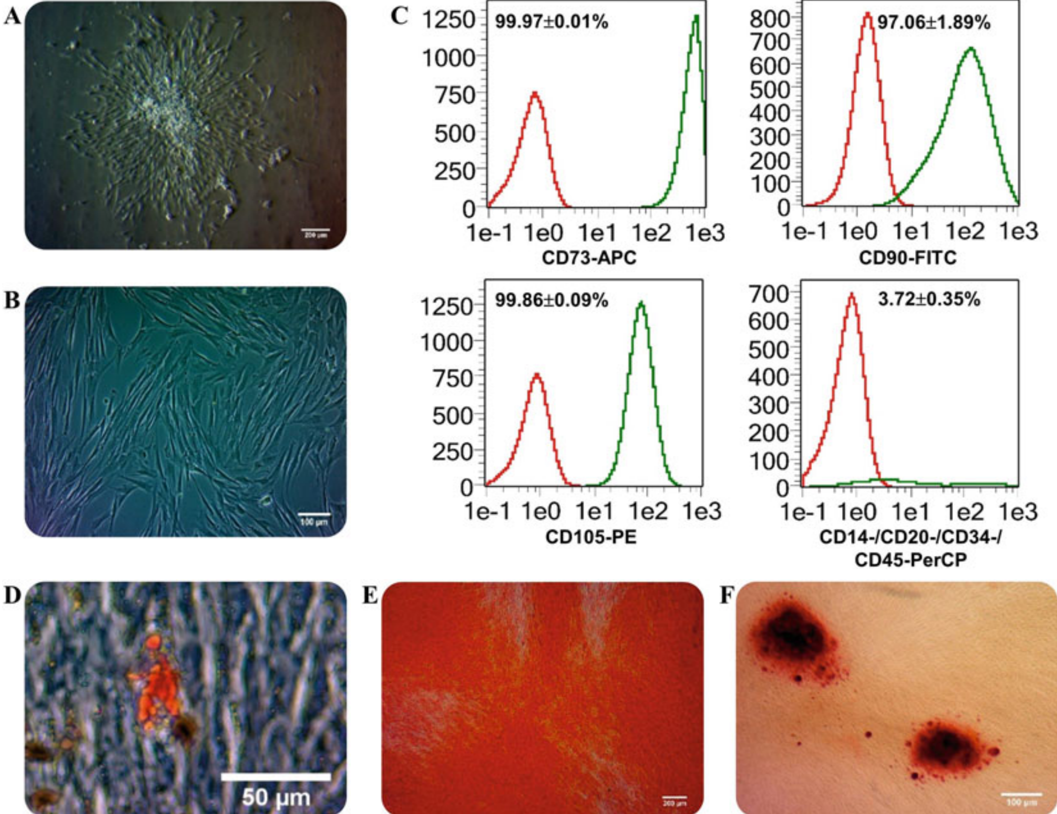

Fig. 2 **MSCs like properties of SHED**. (**a**, **b**) SHED adhere to plastic surfaces. (**c**) Cells were immuno-labelled with a cocktail of fluorochrome-conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD73, fluorescein isothiocyanate (FITC)-conjugated CD90, phycoerythrin (PE)-conjugated CD105, and peridinin-chlorophyll-protein complex (PerCP)-conjugated CD14, CD20, CD34, and CD45. FACS analysis reveals that SHED are positive for CD90, CD105, and CD73 and do not express CD14, CD20, CD34, and CD45. (**d**) Oil Red O positive lipid droplets reveal adipogenic differentiation potential of SHED. (**e**) Safranin O positive staining confirms chondrogenic differentiation potential of SHED, and (**f**) alizarin red positive extracellular calcium deposition indicates osteogenic differentiation potential of SHED

affected by the supplements (Blazquez-Prunera et al. 2017b; Oikonomopoulos et al. 2015). Hence, MSCs like properties of SHED was also studied using the cells expanded in FBS only. SHED with having fibroblast-like morphology, plastic-adhering capacity, tri-lineage differentiation potential, highly expressed (>95%) MSCs positive markers, and negligibly expressed (4% <) MSCs negative markers further confirmed their MSC-like properties (Fig. 2).

Properties of AB blood group specific pHS as an alternative to FBS are frequently being studied in the field of stem cell research (Patrikoski et al. 2013; Aldahmash et al. 2011; Bieback et al. 2012; dos Santos et al. 2017). To widen the donor pool, previously we attempted to prepare pHS from blood irrespective of donors' blood group (Haque and Abu Kasim 2017). In that study, pHS was prepared by pooling serum from six donors to minimize donor-to-donor variation (Haque and Abu Kasim 2017). Another study also reported consistent results among different batches of pHS prepared by pooling sera from six donors (dos Santos et al. 2017). Therefore, in this study, we used the pHS prepared by pooling sera from six donors irrespective to their blood groups to expand SHED prior to analysing their proliferation and paracrine factor expression in the

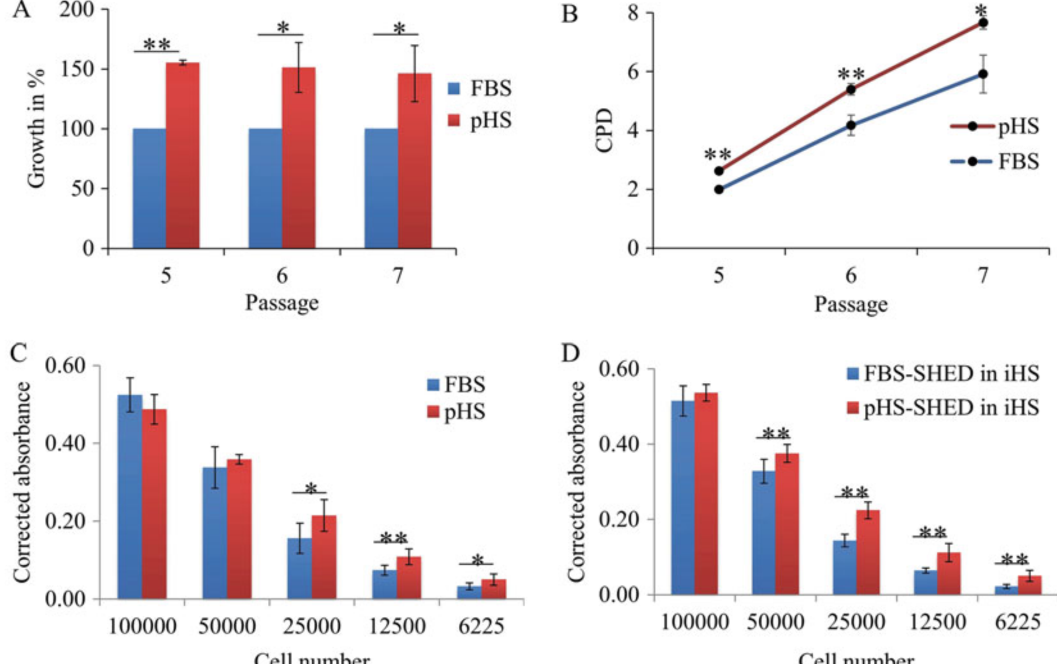

Fig. 3 Proliferation of SHED. (**a**) Comparative growth of SHED ($n = 3$) in pooled human serum (pHS) and foetal bovine serum (FBS) supplemented media. (**b**) Cumulative population doubling (CPD) of SHED in pHS and FBS supplemented media. (**c**) Viability of SHED in the pHS and FBS supplemented media after 24 h of incubation. (**d**) Viability of SHED yielded from pHS and FBS supplemented media (pHS-SHED and FBS-SHED, respectively) in the presence of iHS ($n = 4$) after 24 h of incubation. (* $= p < 0.05$, ** $= p < 0.01$)

presence of iHS. However, to minimize batch-to-batch variations, pooling of sera from a large number of donors could prove more useful (Diez et al. 2015).

Higher proliferation of MSCs in the media supplemented with pHS (prepared from AB blood group-typed donors) has been reported earlier (Turnovcova et al. 2009; Bieback et al. 2012). Recently, we have shown that pHS prepared with sera, regardless of the donors' blood group, maintains higher percentage of rapidly proliferating cells and significantly lower percentage of partially differentiated flat cells (Haque and Abu Kasim 2017). In the present study, pHS-SHED were found to maintain their proliferation potential in iHS supplemented media (Fig. 3). Taken together, these results imply that pHS might represent a suitable supplement for the expansion of SHED prior to transplantation.

Paracrine factors play a vital role in the processes of regeneration by regulating the proliferation, migration, and homing of transplanted cells. In addition to the cell viability, expression of paracrine factors from FBS-SHED and pHS-SHED following exposure to iHS was also studied. By using the same iHS as supplement for both FBS-SHED and pHS-SHED, we have tried to minimize the variations in the composition of paracrine factors in the media. Furthermore, the in vitro and in vivo half-life of paracrine factors is not more than couple of hours (Beutler et al. 1985; Peters et al. 1996). As the supernatants were collected at 24 and 120 h post-incubation, it is expected that the contribution of the paracrine factors in the supernatants by the sera would be negligible or not at all (Haque et al. 2017).

Induced chemotaxis of bone marrow-derived MSCs towards IGF-1, PDGF-BB, and SDF-1a indicates the importance of systemic and local

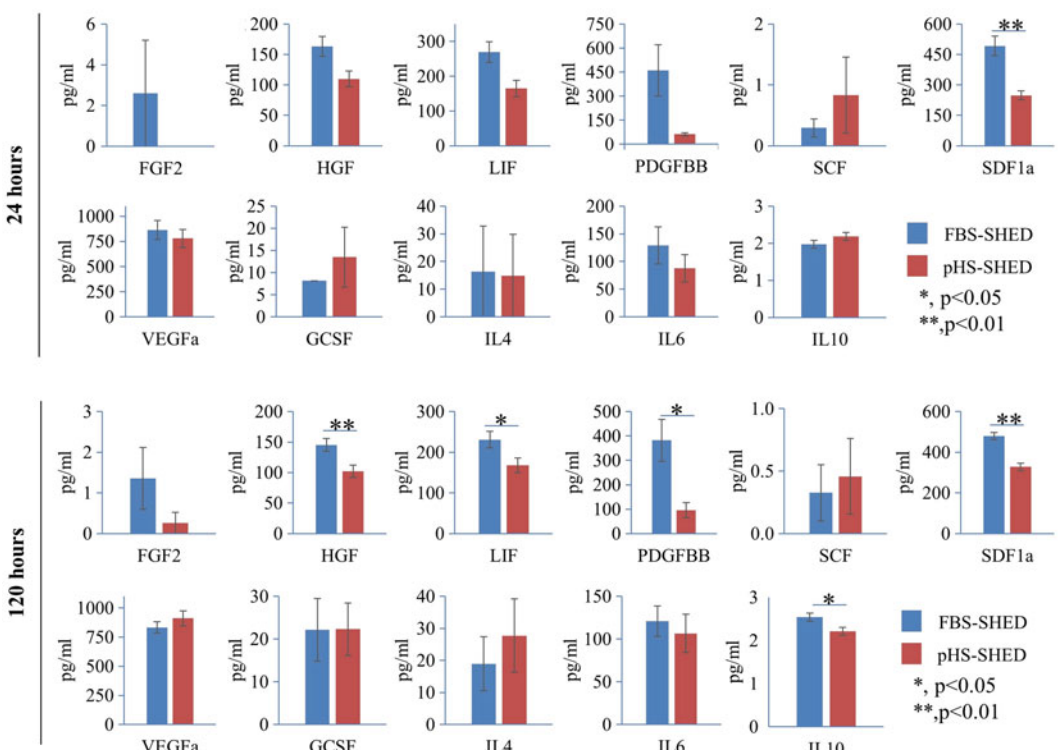

Fig. 4 Comparative expression of paracrine factors in the cell culture supernatants collected from FBS-SHED and pHS-SHED in the presence of freshly prepared individual human serum (iHS) at 24 and 120 h of incubation. (* = $p < 0.05$, ** = $p < 0.01$)

inflammatory state on the migration and homing of cells to the site of injury (Ponte et al. 2007). The role of SDF-1a gradients on the directional migration of MSCs has also been reported earlier (Park et al. 2017). Significantly higher expression of SDF-1 in the supernatants of FBS-SHED collected at both 24 and 120 h of incubation was observed. Significantly higher amount of HGF, LIF, and PDGF-BB in the FBS-SHED supernatants at 120 h of incubation was also measured (Fig. 4). SDF-1, HGF, and PDGF-BB are well-known chemoattractants, and their role in the regenerative therapy has been acknowledged by several researchers (Li et al. 2017; van de Kamp et al. 2017; Ponte et al. 2007). Therefore, higher expression of paracrine factors from FBS-SHED could be a sign of disruption in the gradient of the paracrine factors following transplantation of MSCs expanded in FBS supplemented media that might lead to non-specific engraftment of the transplanted cells.

5 Conclusion

It has been reported that the main shortcoming of MSC-based therapy is low number of engrafted cells affecting the attainment of the long-term functional benefits of this therapy. Gradient of paracrine factors plays a vital role in the tissue-specific migration of transplanted cells. The expression of paracrine factors by FBS-SHED in iHS after 24 and 120 h was higher compared to pHS-SHED. Higher expression of paracrine factors could lead to disruption of the body's own paracrine factor gradient and non-specific engraftment of transplanted and circulatory cells and eventually affect the process of regeneration. Our results indicate that SHED cultivated in human serum instead of FBS have higher proliferative capacity and show an altered secretion profile. Future studies will assess if these differences result in better engraftment and regeneration following transplantation.

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Conflicts of Interest The authors declare no conflicts of interest.

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Molecular Mechanisms Responsible for Anti-inflammatory and Immunosuppressive Effects of Mesenchymal Stem Cell-Derived Factors

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Abstract

Mesenchymal stem cells (MSCs) are self-renewable cells capable for multilineage differentiation and immunomodulation. MSCs are able to differentiate into all cell types of mesodermal origin and, due to their plasticity, may generate cells of neuroectodermal or endodermal origin in vitro. In addition to the enormous differentiation potential, MSCs efficiently modulate innate and adaptive immune response and, accordingly, were used in large number of experimental and clinical trials as new therapeutic agents in regenerative medicine. Although MSC-based therapy was efficient in the treatment of many inflammatory and degenerative diseases, unwanted differentiation of engrafted MSCs represents important safety concern. MSC-based beneficial effects are mostly relied on the effects of MSC-derived immunomodulatory, pro-angiogenic, and trophic factors which attenuate detrimental immune response and inflammation, reduce ischemic injuries, and promote tissue repair and regeneration. Accordingly, MSC-conditioned medium (MSC-CM), which contains MSC-derived factors, has the potential to serve as a cell-free, safe therapeutic agent for the treatment of inflammatory diseases. Herein, we summarized current knowledge regarding identification, isolation, ontogeny, and functional characteristics of MSCs and described molecular mechanisms responsible for MSC-CM-mediated anti-inflammatory and immunosuppressive effects in the therapy of inflammatory lung, liver, and kidney diseases and ischemic brain injury.

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Keywords
Immunosuppression · Inflammatory diseases · Mesenchymal stem cells · Soluble factors · Therapy

Abbreviations

(EMT)	Epithelial-to-mesenchymal transition
AF-MSCs	Amniotic fluid-derived MSCs
AT-MSCs	Adipose tissue-derived MSCs
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BM-MSCs	Bone marrow-derived MSCs
BMP	Bone morphogenetic protein
BPD	Bronchopulmonary dysplasia
C/EBPα	CCAAT/enhancer-binding protein alpha
CCL	CC chemokine ligand
c-MYC	Avian myelocytomatosis virus oncogene cellular homolog
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
ERK	Extracellular signal-regulated kinases
ESCs	Embryonic stem cells
FABP4	Fatty acid-binding protein 4
FAS	Fatty acid synthase
FasL	First apoptosis signal ligand
GLUT4	Glucose transporter type 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hMSCs	Human MSCs
HO-1	Heme oxygenase-1
IDO	Indoleamine 2,3-dioxygenase
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
M-CSF	Monocyte colony-stimulating factor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MSC-CM	MSC-conditioned medium
MSCs	Mesenchymal stem cells
mMSCs	Murine MSCs
MZ	Marginal zone
NECs	Neuroepithelial cells
NK	Natural killer
NKT	Natural killer T cells
NKTregs	Regulatory NKT cells
PAX	Paired box
PGE2	Prostaglandin E2
PL-MSCs	Placenta-derived MSCs
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
RUNX2	Runt-related transcription factor 2
SCF	Stem cell factor
Sox9	Sex-determining region Y-box 9
SSEA	Stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRA-1–60	Tumor resistance antigen 1–60
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells
TSG-6	TNF-α-stimulated gene/protein 6
UC-MSCs	Umbilical cord-derived MSCs

1 Introduction

Stem cells, as self-renewable cells with capacity for pluri- or multilineage differentiation, have raised enormous expectations among healthcare professionals and patients due to their biological importance and therapeutic potential (Volarevic et al. 2011a, 2018). Therapy of many incurable diseases is in the focus of stem cell-based research, and, currently, stem cell-derived tissues, products, and biomaterials represent new hope in regenerative medicine (Volarevic et al. 2011a).

Because of their regenerative and immunomodulatory characteristics, including self-renewability, rapid proliferation, multilineage differentiation, and production of immunosuppressive and pro-angiogenic factors, mesenchymal stem cells (MSCs) are, among stem cells, most usually used in clinical trials as new therapeutic agents for the treatment of inflammatory, degenerative, and ischemic diseases (Gazdic et al. 2017; Markovic et al. 2018; Arsenijevic et al. 2017; Volarevic et al. 2014). MSCs can be easily derived from almost all adult tissues and, accordingly, represent highly accessible cell source with great potential for autologous transplantation (Volarevic et al. 2011b). Moreover, MSCs do not express major histocompatibility complex (MHC) molecules class II and are considered immune-evasive cells capable to engraft in the tissues of MHC-mismatched recipients. Accordingly, MSCs represent valuable cell source for safe allogeneic transplantation (Gazdic et al. 2015). MSC-dependent regeneration of injured tissues is relied on their unlimited differentiation potential. These stem cells differentiate into the cells of mesodermal origin in vivo, while in vitro (under specific culture conditions) MSCs may generate cells of ectodermal and endodermal origin, as well (Volarevic et al. 2011b; Chamberlain et al. 2007). Immediately after transplantation, MSCs are able to migrate toward the site of injury where, through the production of immunomodulatory, pro-angiogenic, and trophic factors, they regulate immune response, induce generation of new blood vessels, and promote tissue repair and regeneration (Volarevic et al. 2017). Since MSCs represent vehicles for the delivery of immunosuppressive and trophic factors, their engraftment attenuates inflammation, encourages endogenous regeneration, and results in repopulation of injured cells (Gazdic et al. 2017; Markovic et al. 2018; Arsenijevic et al. 2017; Volarevic et al. 2014, 2017).

In this chapter we summarized current knowledge about origin, phenotypic, and functional characteristics of MSCs with particular focus on molecular mechanisms which are responsible for beneficial effects of MSC-derived factors in the therapy of chronic inflammatory diseases.

2 Developmental Origin and Characterization of MSCs

Developmental biology has witnessed controversies concerning origin and characterization of MSCs (Fitzsimmons et al. 2018). Most findings support the hypothesis that there are several subpopulations of MSCs that originated from different precursor cells during embryogenesis (Fitzsimmons et al. 2018). Epithelial-to-mesenchymal transition (EMT)-derived cells have a functional resemblance to bone marrow-derived MSCs (BM-MSCs), in terms of antigenic profile, multipotency, and homing capacity, and, accordingly, were proposed as possible precursor cells of MSCs (Battula et al. 2010). Several other studies indicated neural crest origin of MSCs by providing evidence that Sox1+ neuroepithelial cells (NECs) are precursors of MSCs (Takashima et al. 2007a, b; Quirici et al. 2002; da Silva Meirelles et al. 2008). This hypothesis was supported by the findings that MSCs, similar as Sox1 + NECs, expressed receptor for nerve growth factor and were able to differentiate in neuroectodermal (neurons/glias) cells in vitro (Quirici et al. 2002; da Silva Meirelles et al. 2008), while Sox1+ NECs, similar as MSCs, could generate osteoblasts, chondroblasts, and adipocytes (Takashima et al. 2007b). Accordingly, it was suggested that Sox1 + NECs represent the earliest population of MSCs that reside in prenatal tissues, while later in postnatal development, MSCs could be derived

from the cells of nonneural crest origin (Takashima et al. 2007a). In accordance to their multipotency and capacity for spontaneous differentiation in cells of mesodermal origin, lateral plate mesoderm-derived mesoangioblast cells from the embryonic dorsal aorta were proposed as nonneural crest source of MSCs (da Silva Meirelles et al. 2008; Sheng 2015). Additionally, several studies have shown that the blood vessel walls represent an important reservoir of MSC-like stem/progenitor cells (Crisan et al. 2008; Chen et al. 2013). These blood vessel-derived precursor cells, isolated from multiple organs, give rise to cells with typical MSC markers and exhibit capacity for differentiation into osteoblasts, chondrocytes, and adipocytes (Crisan et al. 2008; Chen et al. 2013). Additionally, similarities between MSCs and pericytes in terms of ontogeny, phenotypic and functional characteristics, suggest that these two cell populations originated from the same precursor cell (Harrell et al. 2018a). In line with these findings, it is not an easy task to precisely identify and characterize pure population of MSCs in perivascular tissues (Harrell et al. 2018a).

Having in mind that many diverse antigens have been found on the surface of MSC, but none of them were unique for MSCs (Volarevic et al. 2011a), researchers from International Society for Cellular Therapy focused their attention on morphological and functional properties that were specific for MSCs and managed to define three minimal criteria for characterization of MSCs. First, MSCs have to adhere to plastic culture dishes under standard in vitro conditions. Second, more than 95% of cell population must express CD105 (endoglin, also identified as SH2, a component of the receptor complex of transforming growth factor-beta (TGF-β) involved in proliferation, differentiation, and migration), CD73 (SH3/4, ectoenzyme that regulates the purinergic signaling through the hydrolysis of adenosine triphosphate (ATP)), and CD90 (Thy-1, regulates differentiation of MSCs). MSCs must lack expression of CD45 (pan-leukocyte marker), CD34 (marker of hematopoietic cells), CD14 or CD11b (markers of monocytes), CD79a or CD19 (marker of B lymphocytes), and MHC class II molecules (marker of professional antigen-presenting cells) (Dominici et al. 2006). Third, cells must be able to spontaneously differentiate into adipocytes, osteoblasts, and chondrocytes under standard in vitro differentiating conditions (Dominici et al. 2006). In addition to these well-defined phenotypic and functional characteristics, MSCs constitutively express several adhesion molecules, CD44 (hyaluronan receptor), CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), and CD49b (integrin alpha-2), that enable their migration toward the site of the injury (Dominici et al. 2006).

3 MSC Subpopulations: Phenotype and Functional Characteristics

MSCs reside in perivascular niches of many diverse adult, fetal, and neonatal tissues (bone marrow, adipose tissue, peripheral blood, dental pulp, amniotic fluid, placenta, umbilical cord, etc.) (Hass et al. 2011). Differences in extracellular milieu (influence of neighboring cells and their products, hypoxia) as well as intracellular conditions (expression of certain microRNAs) significantly affect function and therapeutic potential of MSCs. Accordingly, MSCs are considered as heterogeneous group of stem cells that consist of several subpopulations with variable morphological and functional characteristics (Hass et al. 2011).

Among all subpopulations of MSCs, BM-MSCs are best explored and most usually used in experimental and clinical trials. In the bone marrow, MSCs regulate lifelong turnover and growth of bone (Bianco 2014; Wu et al. 2018) and, as major source of stem cell factor (SCF), represent an important cellular component of the hematopoietic stem cell niche (Savickienė et al. 2017). BM-MSC-derived osteocytes promote hematopoiesis, while BM-MSC-derived adipocytes inhibit expansion of hematopoietic progenitors (Bethel et al. 2013). Main biologic

characteristics of BM-MSCs which favor their therapeutic use are rapid proliferation in vitro, reduced expression of MHC molecules and, accordingly, potential for safe allogeneic transplantation, genomic stability after long-term propagation, capacity for spontaneous trilineage (osteogenic, chondrogenic, and adipogenic) differentiation, and suppression of detrimental immune response (Volarevic et al. 2017). However, the derivation of BM-MSCs involves harvesting of bone marrow that is a highly invasive procedure (Nishida et al. 1999; Mueller and Glowacki 2001; Stenderup et al. 2003). Therefore, several alternative tissue sources for isolation of MSCs have been strongly pursued including the adipose tissue, amniotic fluid, umbilical cord, and placenta (Zhou et al. 2014a).

The adipose tissue contains a significant number of MSCs that are easy to harvest by liposuction (Lee et al. 2016). Compared to BM-MSCs, adipose tissue-derived MSCs (AT-MSCs) have similar phenotype, greater proliferation capacity, higher potential for adipogenic differentiation, and inferior potential for osteogenesis and chondrogenesis (Lee et al. 2016; Zuk et al. 2002).

Collection of umbilical cord-derived MSCs (UC-MSCs) is a noninvasive, painless, and safe procedure that has not been encumbered with ethical problems (Nagamura-Inoue and He 2014). MSCs have been isolated from several compartments of the umbilical cord including Wharton's jelly, vein, arteries, UC lining, and subamnion and perivascular regions (Nagamura-Inoue and He 2014). There is no significant difference in the proliferation rate among the cells derived from various compartments of UC, and, importantly, all subpopulations of UC-MSCs exhibit a significantly higher frequency of colony-forming unit fibroblasts than BM-MSCs (Majore et al. 2011; Baksh et al. 2007; Lü et al. 2008). Regarding the differentiation ability, UC-MSCs have higher potential for chondrogenic differentiation than BM-MSCs, but show delayed and insufficient differentiation into osteocytes and adipocytes (Hsieh et al. 2010; Mennan et al. 2013). Interestingly, UC-MSCs may be considered as pluripotent cells, since they express several genes associated with pluripotency: Oct-3/4, Nanog, Sox2, and KLF4 (Greco et al. 2007).

Amniotic fluid-derived MSCs (AF-MSCs) are isolated from amniotic fluid samples obtained through amniocentesis under ultrasonographic control (Tsai et al. 2004; Savickienė et al. 2017; Spitzhorn et al. 2017). Accordingly, amniotic fluid can serve as a rich and advantageous source of MSCs in terms of number of potential donors (Tsai et al. 2004; Moraghebi et al. 2017; Bitsika et al. 2012). Phenotype and differentiation potential of AF-MSCs are similar to both BM-MSCs and embryonic stem cells (ESCs) (De Coppi et al. 2007). AF-MSCs express cell surface antigens CD105, CD90, and CD73 that are expressed on BM-MSCs, and at the same time, AF-MSCs display intracellular and extracellular markers of ESCs, such as Oct-3/4, Nanog, SSEA-3, and SSEA-4, and alkaline phosphatase (Tsai et al. 2004; Moschidou et al. 2013; Joerger-Messerli et al. 2016; Kim et al. 2007; Prusa et al. 2003; Klemmt et al. 2011; Perin et al. 2010). Accordingly, AF-MSCs avian myelocytomatosis virus oncogene cellular homolog (c-MYC), tumor resistance antigen 1–60 (TRA-1–60), and stage-specific embryonic antigen (SSEA) may generate cells of all three germ layers and have notably higher capacity for differentiation than BM-MSCs. It should be highlighted that, in contrast to ESCs, AF-MSCs have stable genotype and are non-tumorigenic in vivo suggesting their potential for safe clinical application (De Coppi et al. 2007; Zhou et al. 2014b).

Several lines of evidence suggest that MSCs derived from placental tissues have superior cell biological properties such as improved proliferative capacity, life span, and differentiation potential than MSCs derived from the bone marrow and other adult tissues. Additionally, ethical concerns related to the derivation of placenta-derived MSCs (PL-MSCs) should be disregarded by the fact that placental tissues are normally considered medical waste and can be recovered without harm to the donor or fetus (Moore et al. 2017). Importantly, PL-MSCs have a higher expansion and engraftment capacity than BM-MSC (Hass et al. 2011), and similar as UC-MSCs and AF-MSCs, PL-MSCs express

pluripotent genes and are able to generate cells of all three germ layers (Hass et al. 2011; Kil et al. 2016; Cho et al. 2018; Jiang et al. 2017).

High-density oligonucleotide microarrays and functional network analyses demonstrated that set of core gene expression profiles has been preserved in all subpopulations of MSCs. This core signature transcriptome includes genes involved in the regulation of osteogenic, adipogenic, and chondrogenic differentiation potential and capacity for immunomodulation (Tsai et al. 2007).

4 Role of Signaling Pathways in Differentiation of MSCs

Signaling pathways involved in differentiation of MSCs toward chondrocytes, adipocytes, and osteocytes have been extensively investigated during the last decade.

Fully differentiated MSC-derived chondrocytes express high levels of runt-related transcription factor 2 (RUNX2), collagen type X, alpha I, and low levels of sex-determining region Y-box 9 (Sox9), which is expressed in early phase of chondrogenesis and is proposed as the main transcription factor responsible for successful differentiation of MSCs into chondrocytes (Akiyama et al. 2002). Members of the TGF-β superfamily (TGF-β1 and bone morphogenetic protein (BMP)) attach to their receptor serine/threonine kinases and activate Smad cascade (Danišovič et al. 2012). Activated Smad1, Smad5, and Smad8 associate with Smad4 and translocate to the nucleus to induce expression of Sox9, collagen type II alpha I, and aggrecan, which are crucially important for the functional properties of chondrocytes (Ikeda et al. 2004; Yu et al. 2012). TGF-β can also induce chondrogenic differentiation of MSCs via the activation of mitogen-activated protein kinase (MAPK) proteins (p38, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinase (JNK)) (Tuli et al. 2003; Zhang 2009; Mu et al. 2012). Unlike TGF-β signaling pathway, the Wnt proteins have been shown to suppress chondrogenic differentiation of MSCs, by reducing expression of SOX9 and collagen type II alpha 1 (Day et al. 2005). In line with these findings, blocking of canonical Wnt signaling in murine as well as human MSCs upregulated expression of collagen type II, alpha 1, and SOX9 and promoted differentiation of MSCs into chondrocytes (Day et al. 2005; Im and Quan 2010).

Key transcriptional factor for adipogenic differentiation of MSCs is peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Rosen et al. 1999). Binding of insulin and insulin-like growth factor 1 (IGF-1) to their receptors play crucial role for the induction of PPAR-γ expression (Muruganandan et al. 2009). Activation of PPAR-γ by insulin/IGF-1 results in increased expression of CCAAT/enhancer-binding protein alpha (C/EBPα), which in turn generates a positive feedback loop and causes further expression of PPAR-γ (Wu et al. 1999). Activated PPAR-γ induces the expression of genes involved in lipid synthesis and storage such as fatty acid synthase (FAS), glucose transporter type 4 (GLUT4), lipoprotein lipase (LPL), and fatty acid-binding protein 4 (FABP4) resulting in the generation of functional adipocytes (Frith and Genever 2008; Oger et al. 2014).

RUNX2 is transcription factor crucially important for differentiation of MSCs into osteocytes (Chen et al. 2014). Activated RUNX2 induce transcription of numerous genes that are important for osteocyte function including collagen type I, alkaline phosphatase, osteocalcin, and bone sialoprotein (Roach 1994; Aubin et al. 1995). However, increased expression of RUNX2 in MSCs is not sufficient to induce generation of functional osteocytes. Accordingly, simultaneous activation of RUNX2 and several other transcriptional factors is needed for successful osteogenic differentiation of MSCs. BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 bind to their receptors and activate Smad1/5/8 as well as ERK, JNK, and p38 kinases of MAPK signaling cascade resulting in increased expression of alkaline phosphatase and osteocalcin in differentiated MSCs (Kang et al. 2009; Dorman et al. 2012; Wang et al. 1993; James 2013; Lai and Cheng 2002). Additionally, RUNX2 may be also a target of Wnt/β-catenin pathway which, through the activation of T-cell factor 1 (TCF1), promotes differentiation of MSCs into functional osteocytes (Gaur et al. 2005).

5 Two-Edged Sword of MSC-Based Immunomodulation

In addition to the potential for multilineage differentiation, MSCs have capacity to modulate innate and adaptive immune response. This crosstalk between MSCs and immune cells within the tissue microenvironment in which MSCs were transplanted is important for modulation of immune cell functions, but is also essential for changes in phenotype and function of MSCs (Gazdic et al. 2015). A huge number of studies showed that MSCs were not constitutively immunosuppressive and that immunomodulatory activities of MSCs were regulated by concentration of inflammatory cytokines secreted by neighboring immune cells.

After engraftment in the tissue with low concentration of inflammatory cytokines (particularly tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ)), MSCs develop inflammatory phenotype and promote host defense to infections (Gazdic et al. 2015; Bernardo and Fibbe 2013). During the onset of inflammation, microbes activate toll-like receptor (TLR)-4 on MSCs and induce polarization of MSCs in pro-inflammatory cells which, in interleukin (IL)-6/signal transducer and activator of transcription (STAT)3, interferon beta (IFN-β), or granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent manner, prevent apoptosis of neutrophils (Raffaghello et al. 2008; Cassatella et al. 2011). Additionally, pro-inflammatory MSCs produce IL-8 and macrophage migration inhibitory factor (MIF) which are responsible for enhanced phagocytic ability and increased recruitment of neutrophils from the circulation into the inflamed tissue. During the early phase of inflammatory response, MSCs might also increase proliferation and activation of T and B lymphocytes (Bernardo and Fibbe 2013; Traggiai et al. 2008; Griffin et al. 2013; Rasmusson et al. 2007a). MSCs primed with low concentrations of IFN-γ and TNF-α secrete CCL5, CXCL9, and CXCL10 which recruit activated T cells to the sites of inflammation and promote T-cell-driven immune response (Bernardo and Fibbe 2013). In tissues where lipopolysaccharides (LPS) or viral antigens induced a weak inflammatory response, MSCs induce expansion and differentiation of B lymphocytes in immunoglobulin-secreting plasma cells and stimulate production of immunoglobulin (Ig)G in paracrine, IL-6-dependent manner, resulting in enhanced humoral immune response (Traggiai et al. 2008; Rasmusson et al. 2007a).

On the contrary, MSCs generate anti-inflammatory phenotype after engraftment in the tissue where inflammatory cytokines, particularly TNF-α and IFN-γ, are present in high concentrations (Gazdic et al. 2015). Enhanced production of these cytokines by inflammatory immune cells promotes generation of immunoregulatory phenotype in MSCs, induces enhanced secretion of MSC-derived immunosuppressive soluble factors, and, accordingly, augments MSC-based suppression of immune response and inflammation (Li et al. 2012; Dazzi and Krampera 2011).

6 MSC-Derived Factors as New Agents in Immunosuppression of Inflammatory Diseases

Most usually, MSCs alter phenotype and function of immune cells in paracrine manner. MSCs, through the production of soluble factors, suppress maturation and antigen-presenting function of dendritic cells (DCs) and macrophages; inhibit proliferation and effector functions of Th1, Th2, and Th17 lymphocytes; attenuate antibody production and class switching in B cells; and suppress cytotoxicity of natural killer (NK) and natural killer T (NKT) cells (Fig. 1).

6.1 Modulation of Antigen-Presenting Cells by MSC-Derived Factors

MSCs, in paracrine manner, affect generation, maturation, proliferation, and capacity for antigen presentation of DCs (Nauta et al. 2006). Among MSC-derived factors, prostaglandin E2 (PGE2), IL-6, and monocyte colony-stimulating factor

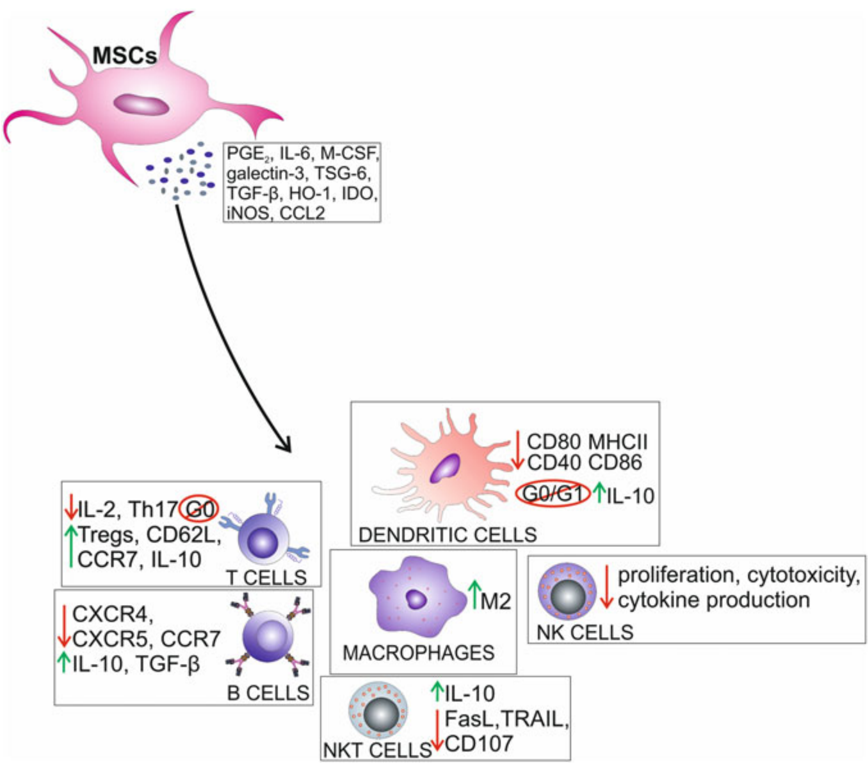

Fig. 1 The effects of MSC-derived factors on phenotype and function of immune cells. Through the production of soluble factors (PGE2, IL-6, galectin 3, TGF-β, PGE2, HO-1, CCL2) and due to the increased iNOS and IDO activity, MSCs induce G0/G1 cell cycle arrest of DCs and T cells, impair antigen presentation ability of DCs and promote their conversion in tolerogenic phenotype, attenuate capacity of plasma cells for antibody production, promote conversion of inflammatory B cells into IL-10+ and TGF-β + regulatory B cells, inhibit proliferation and cytotoxicity of NK and NKT cells, and promote generation of immunosuppressive M2 macrophages, Tregs, and NKTregs

(M-CSF) were considered as the most important molecules for MSC-mediated modulation of DC phenotype and function. MSCs, in IL-6- and M-CSF-dependent manner, inhibit differentiation of DCs from CD34+ progenitor cells and attenuate their proliferation by inducing G0/G1 cell cycle arrest through the decreased expression of cyclin D2 (Nauta et al. 2006; Ramasamy et al. 2007). DCs, cultured with MSCs in transwell systems (which physically separated these two populations), failed to upregulate co-stimulatory molecules CD40, CD80, and CD86 and MHC molecule class II (Zhang et al. 2004; Jiang et al. 2005). These MSC-altered DCs were notably impaired in their ability to present antigen to naïve T cells (Nauta et al. 2006). We recently provided the first evidence that MSCs, through the secretion of immunomodulatory galectin 3, inhibited production of inflammatory cytokines (IL-1β, IL-12, IL-6, and TNF-α) in DCs and attenuated expression of co-stimulatory molecules on their membranes significantly reducing their capacity for activation of naïve T cells (Nikolic et al. 2018). Additionally, MSCs in PGE2-dependent manner suppressed capacity of DCs to induce proliferation and activation of allogeneic T cells (Spaggiari et al. 2009). MSC-derived IL-6 promoted generation of IL-10-producing monocytes (Melief et al. 2013a). Accordingly, MSCs, in IL-6-dependent manner, polarized DCs from inflammatory into tolerogenic, IL-10-producing cells that suppressed cytokine production in effector T cells (Spaggiari et al. 2009; Beyth et al. 2005).

In the inflammatory environment, MSCs were able to actively modulate phenotype of macrophages by suppressing production of inflammatory and by enhancing secretion of immunoregulatory cytokines in these cells (Gazdic et al. 2015). LPS-, IFN-γ-, or TNF-α-primed MSCs, through the production of PGE2, TNF-α-stimulated gene/protein 6 (TSG-6), and IL-6, induced conversion of inflammatory, IL-12- and TNF-α-producing M1 macrophages into anti-inflammatory, alternatively activated M2 cells (Melief et al. 2013a, b; Eggenhofer and Hoogduijn 2012; Németh et al. 2009; Choi et al. 2011; François et al. 2012). M2 macrophages, on turn, through the secretion of IL-10 and CCL18, induced generation of T regulatory cells (Tregs) and enhanced their migration in inflamed tissues, contributing to the creation of immunosuppressive microenvironment (Melief et al. 2013b; Selmani et al. 2008).

6.2 MSC-Derived Factors Suppress Proliferation and Effector Functions of Lymphocytes

Plenty of evidence suggested that MSC-derived soluble factors are crucially important for inhibition of T-cell proliferation. MSCs block interleukin-2-dependent autocrine proliferation in T cells through the secretion of TGF-β, PGE2, and heme oxygenase-1 (HO-1) (Aggarwal and Pittenger 2005; Ghannam et al. 2010a; Bright et al. 1997; Kalinski 2012). These mediators promote G1 cell cycle arrest by suppressing production of IL-2 and by downregulating expression of IL-2 receptor resulting in inhibition of Janus kinase (JAK)-STAT and ERK/MAPK kinase pathways in T cells (Aggarwal and Pittenger 2005; Ghannam et al. 2010a; Bright et al. 1997; Kalinski 2012; Pae et al. 2004). Additionally, in PGE2-dependent manner, MSCs downregulate expression of cyclin D2 and increase expression of the cyclin-dependent kinase inhibitor p27kip1 in T lymphocytes leading to cell cycle arrest (Glennie et al. 2005).

We and others recently demonstrated importance of indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS) for immunosuppressive activity of MSCs (Ren et al. 2009; Gazdic et al. 2018a, b; Milosavljevic et al. 2017). Final products of IDO activity (kynurenine, quinolinic acid, and 3-hydroxyanthranillic acid) negatively affect proliferation or induce apoptosis of T and NKT cells (Ren et al. 2009; Gazdic et al. 2018a, b; Milosavljevic et al. 2017). iNOS generate highly reactive NO which inhibits phosphorylation of STAT5 in T cells, leading to the cell cycle arrest (Sato et al. 2007). Ren and colleagues suggested that MSC-based modulation of T-cell-dependent immune response varied among species (Ren et al. 2009). They proposed that human MSCs (hMSCs) suppress proliferation and effector functions of T cells in IDO-dependent manner, while murine MSCs (mMSCs) overexpress iNOS and, through the production of NO, inhibit expansion and activation of T lymphocytes (Ren et al. 2009). Nevertheless, NO is highly unstable, it only acts locally (Sato et al. 2007) and, accordingly, could not be responsible for systemic and endocrine effects of mMSC-based therapy. By using animal model of acute liver injury, we recently demonstrated that under Th1 inflammatory conditions (in the presence of elevated levels of IFN-γ), mMSCs initially produce NO which, in autocrine manner, induces increased IDO activity in mMSCs, resulting with enhanced production of kynurenine that suppresses proliferation and effector functions of NKT cells (Gazdic et al. 2018b). Importantly, the same molecular mechanism (interplay between NO and IDO) was crucially important for hMSC-mediated suppression of activated human peripheral blood lymphocytes (Gazdic et al. 2018b).

In line with these findings, we demonstrated that NO- and IDO-dependent attenuation of acute liver injury in MSC-treated mice was accompanied with reduced presence of liver-infiltrated inflammatory (IFN-γ- and IL-17-producing) NKT cells and with an increased influx of immunosuppressive IL-10-producing CD4 + CD25 + FoxP3+ Tregs and FoxP3+ regulatory NKT cells (NKTregs) in the injured livers (Gazdic et al. 2018a; Milosavljevic et al. 2017). MSCs managed to prevent expansion of

inflammatory IL-17-producing Th17 and NKT17 cells by promoting their conversion in immunosuppressive Tregs and NKTregs (Gazdic et al. 2018a; Milosavljevic et al. 2017; Duffy et al. 2011; Ghannam et al. 2010b). Additionally, MSCs, in paracrine, NO- and IDO-dependent manner, significantly increased expression of CD62L and CCR7 and increased production of immunosuppressive IL-10 and TGF-β in Tregs enhancing their migratory and immunosuppressive capacities (Gazdic et al. 2018a). In similar, NO- and IDO-dependent manner, MSCs suppressed expansion of NKT cells, reduced expression of apoptosis-inducing ligands (first apoptosis signal ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)), downregulated expression of CD107 (marker of degranulation), and polarized NKT cells from inflammatory, TNF-α-, IFN-γ-, and IL-17-producing cells into immunosuppressive, IL-10-producing cells contributing to the attenuation of NKT cell-dependent cytotoxicity and inflammation (Milosavljevic et al. 2017; Gazdic et al. 2018b). 1-Methyl-DL-tryptophan (specific IDO inhibitor) or L-NG-monomethyl arginine citrate (specific iNOS inhibitor) completely abrogated immunoregulatory capacity of MSCs in vitro and in vivo and restored pro-inflammatory cytokine production and cytotoxicity of NKT cells, suggesting that increased activity of iNOS and IDO was crucially important for MSC-mediated suppression of NKT cells (Milosavljevic et al. 2017; Gazdic et al. 2018b).

MSCs efficiently modulated proliferation, activation, and cytotoxicity of NK and cytotoxic T lymphocytes (CTLs) in paracrine manner. MSC-derived IDO, PGE2, and TGF-β1 were able to reduce expression of the activating receptors (NKp30, NKp44, and NKG2D), attenuate cytotoxicity, inhibit production of inflammatory cytokines (IFN-γ, TNF-α), and suppress IL-2-dependent proliferation of CTLs and NK cells (Rasmusson et al. 2007b; Li et al. 2014; Sotiropoulou et al. 2006; Spaggiari et al. 2008).

In line with these findings, MSCs, in paracrine, NO- and IDO-dependent manner, suppressed influx of IL-6- and TNF-α-producing, inflammatory B cells in the liver and increased presence of liver-infiltrated immunosuppressive (IL-10- and TGF-β-producing) marginal zone (MZ)-like regulatory B cells (CD23-CD21 + IgM+) resulting in the significant attenuation of acute liver inflammation (Gazdic et al. 2018a). Additionally, through the activation of IDO/kynurenine pathway, MSCs induce apoptosis of B cells (Corcione et al. 2006) and block ERK1/2 phosphorylation resulting in division arrest and anergy of B cells (Tabera et al. 2008). MSCs downregulate expression of chemokine receptors (CXCR4, CXCR5, and CCR7) on naïve and activated B cells, affecting their homing and migratory capacities (Corcione et al. 2006), but did not alter expression of co-stimulatory molecules (CD80 and CD86) on B cells and, accordingly, did not reduce their capacity for antigen presentation (Corcione et al. 2006). MSC-derived CC chemokine ligand (CCL)2 suppresses immunoglobulin production in plasma cells by modulating expression of paired box (PAX)5 and STAT3 (Rafei et al. 2008).

7 MSC-CM as New, Cell-Free Therapeutic Agent in Regenerative Medicine

Despite the fact that MSCs have proved their therapeutic potential in a large number of studies, unwanted differentiation of engrafted MSCs in vivo is still the most important safety concern related to MSC-based immunomodulation and regeneration (Volarevic et al. 2018). Various growth factors, produced in the tissue microenvironment where MSCs are engrafted, may induce spontaneous and unwanted differentiation of MSCs toward the cells of mesodermal origin, most usually the cartilage and bone (Volarevic et al. 2018). Accordingly, a large number of studies investigated immunomodulatory and regenerative potential of MSC-CM, as MSC-derived cell-free therapeutic agent which can bypass many of the limitations of MSC-based therapy, including safety concern related to unwanted differentiation of engrafted MSCs (Table 1).

MSC-CM contains broad range of MSC-derived immunomodulatory factors, and, accordingly, injection of MSC-CM showed

Table 1 Therapeutic potential of MSC-CM

MSC-CM as a cell-free therapeutic agent		
Disease	Outcomes	Mechanism of action
Acute lung injury (Ionescu et al. 2012a; Lee et al. 2009)	Improvement of lung endothelial barrier, reduction of pulmonary edema, suppression of inflammatory response	Insulin-like growth factor, keratinocyte growth factor
Bronchopulmonary dysplasia (Monsel et al. 2016)	Prevention of blood vessel remodeling, reduction of neutrophil and macrophages influx in the lungs, decreased expression of pro-inflammatory cytokines (IL-6 and IL-1β)	Macrophage-stimulating factor 1, osteopontin, stanniocalcin-1
Chronic inflammatory lung diseases (Ionescu et al. 2012b; Abreu et al. 2017; Harrell et al. 2018b)	Prevention of airway hyperresponsiveness, reduction of peribronchial inflammation and airway remodeling, attenuated production of Th2 cytokines (IL-4 and IL-13), expansion of Tregs and M2 macrophages	Adiponectin exosomes containing IL-1Ra, IL-27, CXCL14, CXCL16
Acute liver injury (Gazdic et al. 2018a, b; Huang et al. 2016; Milosavljevic et al. 2017; Parekkadan et al. 2007; van Poll et al. 2008; Xagorari et al. 2013)	Suppression of hepatocyte apoptosis, attenuation of liver inflammation, reduced hepatotoxicity and total number of IFN-γ+ and IL-17+ NKT cells, increased presence of IL10+ Tregs and NKTregs	IL-6, fibrinogen-like protein 1, IDO
Liver fibrosis (Milosavljevic et al. 2018)	Expansion of protective FoxP3 + IL-10+ Tregs, suppressed activation of pro-fibrogenic Th17 cells and stellate cells	IDO
Acute kidney injury (Bi et al. 2007; Liu et al. 2018; Simovic Markovic et al. 2017; Overath et al. 2016)	Decreased serum levels of creatinine, IL-1, and IL-6; decreased influx of activated neutrophils, TNF-α + DCs, and IL-17+ CTLs in the kidneys; increased presence of tolerogenic DCs and Tregs	iNOS
Ischemic brain injury (Jiang et al. 2018; Egashira et al. 2012; Faezi et al. 2018)	Recovery of motor functions, reduction of infarct volume and brain edema	Tissue inhibitor of metalloproteinase-1 (TIMP-1) progranulin insulin-like growth factor, brain-derived neurotrophic factor

beneficial effects in the treatment of inflammatory lung, liver, and kidney diseases (Pierro et al. 2013; Ionescu et al. 2012a, b; Lee et al. 2009; Monsel et al. 2016; Aslam et al. 2009; Sutsko et al. 2013; Abreu et al. 2017; Cruz et al. 2015; Du et al. 2018; Harrell et al. 2018b; Ortiz et al. 2007; Tan et al. 2018; van Poll et al. 2008; Xagorari et al. 2013; Parekkadan et al. 2007; Huang et al. 2016; Milosavljevic et al. 2018; Bi et al. 2007; Liu et al. 2018; Simovic Markovic et al. 2017; Overath et al. 2016). Additionally, due to the presence of trophic and pro-angiogenic factors, MSC-CM efficiently protected brain tissue from ischemic injury (Jiang et al. 2018; Egashira et al. 2012; Faezi et al. 2018).

MSC-CM administration exerts short- as well as long-term therapeutic effects in immune-mediated lung injury (Pierro et al. 2013). Intratracheal application of MSC-CM (concentrated 25x) significantly reduced pulmonary edema and inflammation in animal model of LPS-induced acute lung injury (Ionescu et al. 2012a). MSC-derived IGF-1, contained in MSC-CM, induced conversion of macrophages

toward immunosuppressive M2 phenotype resulting in notable attenuation of inflammation (Ionescu et al. 2012a), while MSC-derived keratinocyte growth factor (KGF) was crucially important for the regenerative effects of MSC-CM (Lee et al. 2009). In similar manner, allogeneic human MSC-CM improved lung endothelial barrier and restored alveolar fluid clearance in an ex vivo perfused human lungs injured by LPS (Lee et al. 2009).

Several studies confirmed beneficial effects of MSC-CM in a murine model of bronchopulmonary dysplasia (BPD) (Monsel et al. 2016). MSC-CM prevented blood vessel remodeling and alveolar injury and significantly reduced influx of neutrophils and macrophages in the lungs of hyperoxia-exposed mice (Aslam et al. 2009). MSC-CM-based improvement of lung structure in hyperoxic pups was associated with decreased expression of pro-inflammatory cytokines (IL-6 and IL-1β), followed by attenuation of ongoing inflammation (Sutsko et al. 2013). Macrophage-stimulating factor 1, osteopontin, and antioxidant stanniocalcin-1, all present at high levels in MSC-CM, were responsible for beneficial immunomodulatory and therapeutic effects of MSC-CM in the therapy of experimental BPD (Aslam et al. 2009).

MSC-CM efficiently attenuated acute and chronic asthma in several experimental studies (Ionescu et al. 2012b; Abreu et al. 2017). MSC-CM attenuated production of Th2 cytokines (IL-4 and IL-13) and promoted expansion of IL-10-producing Tregs and M2 macrophages contributing to the creation of anti-inflammatory microenvironment within the asthmatic lungs (Ionescu et al. 2012b). Adiponectin, an anti-inflammatory adipokine found in MSC-CM, was suggested as important MSC-derived factor for the prevention of airway hyperresponsiveness, peribronchial inflammation, and airway remodeling (Ionescu et al. 2012b).

Cruz and co-workers suggested that MSC-derived exosomes (nano-sized extracellular vesicles that deliver proteins, lipids, DNA fragments, and microRNA to the tissue-resident and immune cells) were responsible for attenuation of airway allergic inflammation (Cruz et al. 2015). Similar conclusions were drawn by Du and colleagues who confirmed that MSC-derived exosomes alleviated airway inflammation, enhanced proliferation and immunosuppressive properties of Tregs, and enhanced production of anti-inflammatory cytokines (IL-10 and TGF-β) in peripheral blood mononuclear cells obtained from asthmatic patients (Du et al. 2018). In line with these results are our findings related to the therapeutic potential of "Exosomes d-MAPPS," which activity was based on PL-MSC-derived exosomes containing interleukin 1 receptor antagonist (IL-1Ra) and several other immunomodulatory cytokines and chemokines (IL-27, CXCL14, CXCL16) (Harrell et al. 2018b). When MSC-derived IL-1Ra binds to the IL-1 receptor (IL-1R) on lung epithelial cells, various pro-inflammatory events initiated by IL-1:IL-1R binding become inhibited (including the synthesis and releases of inflammatory cytokines that attract neutrophils, macrophages, and lymphocytes in injured lungs), resulting in the attenuation of lung inflammation (Ortiz et al. 2007). Accordingly, results, obtained in a pilot trial with small number of patients, revealed notably attenuated lung inflammation and significantly improved pulmonary function parameters in Exosomes d-MAPPS-treated patients with chronic lung inflammation (Harrell et al. 2018b). Similar results, related to the efficacy of MSC-derived exosomes in the therapy of lung injury and fibrosis, were obtained by Tan and co-workers who found that MSC-derived exosomes attenuated fibrosis, recovered pulmonary function, and enhanced endogenous lung repair (Tan et al. 2018).

Similar as it was observed in MSC-based therapy of lung inflammation, MSC-CM efficiently attenuated inflammation and fibrosis in the liver (Gazdic et al. 2017). MSC-CM dramatically reduce total number and cytotoxicity of liver-infiltrated immune cells, attenuated apoptosis of hepatocytes, and increased their proliferation resulting in significantly improved survival of MSC-CM-treated animals (van Poll et al. 2008; Xagorari et al. 2013; Parekkadan et al. 2007;

Huang et al. 2016). Several lines of evidence demonstrated that IL-6 and fibrinogen-like protein 1 were responsible for MSC-CM-based suppression of apoptosis and enhanced regeneration of hepatocytes, while enhanced activity of IDO/kynurenine pathway was mainly responsible for MSC-CM-mediated attenuation of detrimental immune response in the liver (Gazdic et al. 2017, 2018a, b; Milosavljevic et al. 2017; Xagorari et al. 2013; Parekkadan et al. 2007). MSC-CM significantly reduced hepatotoxicity and total number of inflammatory IFN-γ- and IL-17-producing NKT cells and notably increased presence of FoxP3 + IL-10+ Tregs and NKTregs cells in the livers of mice with acute hepatitis (Milosavljevic et al. 2017). This phenomenon was completely abrogated in the presence of IDO inhibitor, confirming that IDO/kynurenine pathway was responsible for MSC-CM-based suppression of acute liver inflammation (Milosavljevic et al. 2017). Similar cellular and molecular mechanisms were involved in MSC-CM-mediated attenuation of liver fibrosis. MSC-CM promoted expansion of protective FoxP3 + IL-10+ Tregs and suppressed activation of pro-fibrogenic Th17 cells and stellate cells in IDO-/kynurenine-dependent manner (Milosavljevic et al. 2018).

MSC-CM significantly reduced apoptosis of tubular cells, improved renal function, and increased survival of mice suffering from acute kidney injury and renal fibrosis (Bi et al. 2007; Liu et al. 2018). MSC-CM efficiently attenuated cisplatin-induced nephrotoxicity by reducing the influx and capacity of DCs and T lymphocytes to produce inflammatory cytokines (Simovic Markovic et al. 2017). NO was mainly responsible for MSC-CM-mediated renoprotective effects since inhibition of iNOS activity in MSCs and, accordingly, lack of NO in MSC-CM resulted in increased influx of inflammatory, TNF-α-producing DCs and IL-17-producing CTLs and decreased presence of IL-10-producing tolerogenic DCs and Tregs in cisplatin-treated mice (Simovic Markovic et al. 2017). In line with these findings, Overath and co-workers showed that injection of MSC-CM significantly attenuated cisplatin-induced acute kidney injury and inflammation as demonstrated by downregulated serum levels of creatinine, IL-1, and IL-6 and reduced presence of activated neutrophils in injured kidneys (Overath et al. 2016).

MSC-CM-based therapy protected brain tissue from ischemic injury and promoted functional recovery after stroke in experimental mice and rats (Jiang et al. 2018; Egashira et al. 2012). Intracerebroventricular administration of MSC-CM markedly reduced infarct volume and brain edema in tissue inhibitor of metalloproteinase-1 (TIMP-1) and progranulin-dependent manner and in IGF-1 and brain-derived neurotrophic factor (BDNF)-dependent manner significantly improved cognitive and motor skills (Egashira et al. 2012). MSC-CM notably reduced neuronal loss by affecting expression of caspase-3, Bax, and Bcl-2 in motor cortex and accordingly resulted in recovery of motor functions in experimental animals (Faezi et al. 2018).

8 Conclusions

Due to their immunomodulatory and regenerative abilities, MSCs and their secretomes represent potentially new therapeutic agents in regenerative medicine. Although MSC-based therapy was efficient in the treatment of many inflammatory and degenerative diseases, unwanted differentiation of engrafted MSCs represents important safety concern. Beneficial immunoregulatory effects of MSCs are mainly relied on the effects of their soluble factors which act through multiple mechanisms affecting maturation, phenotype, and function of naïve and effector immune cells of innate and acquired immunity. Accordingly, MSC-CM has the potential to serve as a cell-free therapeutic agent for the treatment of immune cell-mediated diseases. However, it should be noted that MSC-CM contains broad number of immunosuppressive and pro-angiogenic factors, and its therapeutic use could inhibit immune surveillance of tumor cells and may promote uncontrolled growth and expansion of tumor cells. Accordingly, previous history of malignant

diseases has to be considered as an important exclusion criteria for the use of MSC-CM. In line with these observations, new experimental and clinical studies have to determine the exact protocols for therapeutic application of MSC-CM and should focus their attention on long-term safety issues related to MSC-CM-based therapy before this MSC-derived product could be broadly used in regenerative medicine.

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Evaluation of Proliferation and Osteogenic Differentiation of Human Umbilical Cord-Derived Mesenchymal Stem Cells in Porous Scaffolds

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Abstract

Introduction: Human umbilical cord-derived mesenchymal stem cells (UCMSCs) are multiple potential stem cells that can differentiate into various kinds of functional cells, including adipocytes, osteoblasts, and chondroblasts. Thus, UCMSCs have recently been used in both stem cell therapy and tissue engineering applications to produce various functional tissues. This study aimed to evaluate the proliferation and differentiation of UCMSCs on porous scaffolds.

Methods: UCMSCs were established in a previous study and kept in liquid nitrogen. They were thawed and expanded in vitro to yield enough cells for further experiments. The cells were characterized as having MSC phenotype. They were seeded onto culture medium-treated porous scaffolds or on non-treated porous scaffolds at different densities of UCMSCs (10^5, 2.1×10^5, and 5×10^5 cells/0.005 g scaffold). The existence of UCMSCs on the scaffold was evaluated by nucleic staining using Hoechst 33342 dye, while cell proliferation on the scaffold was determined by MTT assay. Osteogenic differentiation was evaluated by changes in cellular morphology, accumulation of extracellular calcium, and expression of osteoblast-specific genes (including *runx2*, *osteopontin* (*OPN*), and *osteocalcin* (*OCN*)).

Results: The data showed that UCMSCs could attach, proliferate, and differentiate on

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both treated and non-treated scaffolds but were better on the treated scaffold. At a cell density of 10^5 cells/0.005 g scaffold, the adherent and proliferative abilities of UCMSCs were higher than that of the other densities after 14 days of culture ($p < 0.05$). Adherent UCMSCs on the scaffold could be induced into osteoblasts in the osteogenic medium after 21 days of induction. These cells accumulated calcium in the extracellular matrix that was positive with Alizarin Red staining. They also expressed some genes related to osteoblasts, including *runx2*, *OPN*, and *OCN*.

Conclusion: UCMSCs could adhere, proliferate, and differentiate into osteoblasts on porous scaffolds. Therefore, porous scaffolds (such as Variotis) may be suitable scaffolds for producing bone tissue in combination with UCMSCs.

Keywords
3D porous scaffold · Osteogenic differentiation · UCMSCs · Variotis

Abbreviations

ECM	Extracellular matrix
HAc	Hyaluronic acid
OCN	*Osteocalcin*
OPN	*Osteopontin*
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
UCMSCs	Umbilical cord-derived mesenchymal stem cells

1 Introduction

Bone tissue is one tissue that is capable of repairing itself through the bone remodeling process (Robling et al. 2006). However, the normal remodeling process is slow and cannot keep up with the repair of excessive damages (David et al. 2007). This leads to an increase of the need for bone graft to treat bone diseases, trauma, and bone cancer (Wang et al. 2014). Although autogenous bone graft is the standard treatment, this method has several limits, including donor site morbidity and constraints on obtainable quantities (Mishra et al. 2016). Allogeneic bone graft is considered to be an alternative treatment method. However, it is hampered by minor immunogenic rejection, disease transmission, and lack of blood supply (Holzmann et al. 2010). Therefore, artificial bone tissue engineering represents a promising therapy to meet the aforementioned unresolved issues. The field of bone tissue engineering has emerged recently as a convenient alternative to facilitate the regenerative ability of host tissues (Amini et al. 2012).

Umbilical cord-derived mesenchymal stem cells (UCMSCs) have been demonstrated to have the capacity to differentiate into multiple cell lineages in all three embryonic germ layers (Wang et al. 2009) and can be harvested at a low cost without an invasive procedure (Chen et al. 2013). Moreover, several reports have indicated that human UCMSCs exhibit potential of osteogenic differentiation on three-dimensional scaffolds (Wang et al. 2010; Ahmadi et al. 2017). Osteogenic differentiation of MSCs takes place at different stages, with each stage characterized by the expression of specific genes (Bruderer et al. 2014). In the early stages, there is a strong proliferation of cells. Then, the growth tends to decrease as the differentiation process begins (Cooper 2000).

There are many vital transcription factors for osteoblast differentiation. Among of them, *runx2* is a main determinant of osteoblast differentiation and controls bone formation (Wang et al. 2010; Fakhry et al. 2013). *Runx2* belongs to the *runx* family of transcription factors and regulates osteoblast differentiation (Ziros et al. 2008). It is strongly expressed in the early stages of osteoblast differentiation, particularly in the first week of differentiation (Fakhry et al. 2013). The expression of *runx2* leads to the upregulation of crucial genes in the osteoblast differentiation, such as *osteocalcin*, *alkaline phosphatase*, *bone sialoprotein*, and *osteopontin* (Ducy et al. 1997). *Osteocalcin* (*OCN*) constitutes 1–2% of the matrix proteins and is the most abundant non-collagenous protein that is exclusively secreted by osteoblasts (Lian et al. 1989). *OCN*

controls the size and speed of bone formation (Roach 1994). *Osteopontin* (*OPN*) is another important non-collagenous protein in the bone matrix that is involved in bone remodeling (McKee and Nanci 1996). *OPN* contains the Arg-Gly-Asp sequence as the cell-binding motif; this motif links the adhesion molecules of cells to the extracellular matrix in the mineralized bone matrix (Roach 1994; Oldberg et al. 1986). *OCN* and *OPN* are expressed in mature osteoblasts only and are absent at the early stages; thus, they are markers of late osteoblast differentiation (Wang et al. 2010; Rutkovskiy et al. 2016). In short, the expression of these genes indicates that MSCs have differentiated into osteoblasts.

There are various scaffolds that have been used in bone engineering including natural polymers scaffolds, such as collagen (Aravamudhan et al. 2013), chitosan (Costa-Pinto et al. 2011), silk fibroin (Vepari and Kaplan 2007), and hyaluronic acid (HAc) (Pavasant et al. 1994). Synthetic polymers scaffolds like polyesters (e.g., polyglycolic acid, polylactic acid, polycaprolactone) are the most commonly used as copolymers. Compared to these scaffolds, the Variotis scaffold (Biometic, Sydney, Australia) is a novel scaffold that is comprised of a highly interconnected and porous structure (> 95%). Based on the unique pore structure, the Variotis scaffold is a suitable scaffold for both soft and hard tissue regeneration applications. The scaffold is made from a polyester-based material that is used to enhance cell attachment and proliferation (Zhang et al. 2013). Previously, the scaffold was used to repair cartilage lesion and heal wounds (Ark et al. 2016; Zhang et al. 2015). To date, the Variotis scaffold shows potential for applications that promote cell growth, as well as vascular and extracellular matrix (ECM) formation. Therefore, it is a promising scaffold for tissue engineering (Zhang et al. 2013).

In this study, we aimed to investigate the adherence and proliferation of UCMSCs on the porous scaffold. Morphological changes, calcium extracellular deposition, and specific gene expression were also surveyed after osteogenic differentiation.

2 Materials-Methods

2.1 Porous Scaffold

Porous scaffolds (Variotis, Biometic, Sydney, Australia) with a pore size in excess of 100 μm were weighed using a balance to reach 0.005 g. The scaffolds samples were divided randomly into two groups: treated and non-treated. For the pretreated group, scaffold samples were immersed in culture medium for 24 h.

2.2 Characteristics of Human Umbilical Cord Mesenchymal Stem Cells

The UCMSCs were isolated per previously published and cryopreserved (Van Pham et al. 2016). The cryopreserved UCMSCs were thawed following the protocol of Pham Van Phuc et al. (Van Pham et al. 2016). Briefly, the vials were placed in a water bath at 37 °C for 1–2 min, and then thawing medium was added and centrifuged at 100 g for 5 min to collect the cell pellet. The pellet was resuspended with 3 mL MSCCult medium (Regenmedlab, Ho Chi Minh City, Vietnam) and cultured in a T-25 flask in an incubator at 37 °C, 5% CO_2.

UCMSCs were characterized as mesenchymal stem cells based on the minimal criteria of MSCs, as suggested by the International Society for Cellular Therapy (ISCT), which includes cellular morphology, marker profiles, and differentiation potential. For marker profiles, the expression of certain markers of UCMSCs was evaluated by flow cytometry per published protocol (Van Pham et al. 2016). Briefly, UCMSCs were suspended in staining buffer at 10^4 cells/100 µl in a tube. Then, each tube was stained with specific antibodies, such as CD14-FITC (Santa Cruz Biotechnology, Dallas, Texas), CD34-FITC (Santa Cruz Biotechnology), CD73-FITC (Santa Cruz Biotechnology), CD90-FITC (Santa Cruz Biotechnology), CD44-APC (Sigma), and HLA-DR-FITC (BD Biosciences) for 30 min in the

dark at room temperature. Stained cells were washed with PBS twice to remove the extra antibodies, and then analysis of marker expression was conducted using an FACSCalibur instrument (BD Biosciences, Franklin Lakes, New Jersey) and compared to unstained and isotype controls.

For in vitro differentiation, UCMSCs were induced to several kinds of mesoderm cells, including adipocytes, osteoblasts, and chondroblasts, in the inducing medium (StemPro™ Adipogenesis Differentiation Kit, StemPro™ Osteogenesis Differentiation Kit, StemPro™ Chondrogenesis Differentiation Kit, all bought from Thermo Fisher Scientific, Waltham, MA). For adipocyte differentiation, after 14 days of induction, the medium was removed, and cells were washed with phosphate-buffered saline (PBS) and fixed in paraformaldehyde (PFA), 4% for 1 h. The cells were washed again with PBS and stained with Oil Red dye (Sigma-Aldrich, Louis St., MO). For chondroblast differentiation, after 21 days of induction, cells were also washed with PBS and stained with Alcian Blue (Sigma-Aldrich, Louis St., MO) to detect proteoglycan deposition. For osteoblast differentiation, after 21 days of induction, cells were washed twice with PBS and then stained with Alizarin Red dye (Sigma-Aldrich, Louis St., MO) to detect accumulation of extracellular calcium.

2.3 Seeding UCMSCs onto Porous Scaffold

UCMSCs were expanded in the flask until they reached approximately 80% confluency. They were then dissociated with trypsin/EDTA, 0.25% (Sigma-Aldrich, Louis St., MO). UCMSCs were suspended in culture medium at different cell densities (10^5, 2×10^5 and 5×10^5 cells, per 0.005 g scaffold). Cell suspensions were directly seeded onto the two groups of scaffolds: pretreated and un-pretreated scaffolds (in 15 ml Falcons). Then, the cell-seeded scaffolds were cultured at 37 °C, 5% CO_2.

2.4 Evaluation of Cell Proliferation on the Scaffold

Cell proliferation was evaluated on the scaffold by observation under a microscope and by MTT assay. UCMSCs on scaffolds were observed after 3, 7, 14, and 21 days of seeding, via a microscope. Proliferation of the cells was assessed by MTT assay at 3, 7, and 14 days. For all assays, non-seeded scaffolds were used as a negative control.

In the MTT assay, culture medium was removed. Then, 500 μl fresh medium and 50 μl MTT reagent (5 mg/ml) were added into each well. The samples were incubated at 37 °C for 4 h. MTT-containing medium was replaced by 500 μL DMSO solution (Sigma-Aldrich, Louis St., MO). The samples were vortexed, and the absorbance at 595 nm was recorded by a DTX-880 system (Beckman Coulter, Brea, CA).

2.5 Osteogenic Differentiation of Seeded Scaffolds

Seeded scaffolds were cultured for 14 days to expand the UCMSCs on the surface. Then, the culture medium was replaced by the osteogenic medium (StemPro™ Osteogenesis Differentiation Kit, Thermo Fisher Scientific, Waltham, MA) for 28 days and refreshed with osteogenic medium every 4 days. The osteogenic differentiation of UCMSCs on the scaffolds was evaluated based on (1) morphology, (2) deposition of extracellular calcium, and (3) osteoblast-specific gene expression.

Changes in cellular morphology were observed after 7, 14, 21, and 28 days of induction. After 21 and 28 days of induction, cell-seeded scaffolds were stained with Alizarin Red dye to detect deposition of extracellular calcium.

The expression of certain genes related to osteoblasts was investigated; these included *Runx2*, *OCN*, *OPN*, and *β-actin*, at four time points after induction (7, 14, 21, and 28 days). UCMSCs on scaffolds in both groups were detached by Detachment solution (Regenmedlab,

Table 1 Primer sequences of the specific genes for osteogenic differentiation

Gene	Primer sequence (5′ to 3′)		T_m (°C)	Product size (bp)	GenBank no.
β-Actin	Forward	AGAGCTACGAGCTGCCTGAC	54,5	184	NM_001101.4
	Reverse	AGCACTGTGTTGGCGTACAG			
OCN	Forward	GTGACGAGTTGGCTGACC	53,3	114	NM_1991735
	Reverse	TGGAGAGGAGCAGAACTGG			
OPN	Forward	GACACATATGATGGCCGAGGTGATAG	59	111	NM_001251830.1
	Reverse	GGTGATGTCCTCGTCTGTAGCATC			
Runx2	Forward	GGAGTGGACGAGGCAAGAGTTT	54,5	133	NM_001278478.1
	Reverse	AGCTTCTGTCTGTGCCTTCTGG			

HCM City, Vietnam). The cell pellets were used to isolate total RNA by easy-BLUE Total RNA Extraction Kit (iNtRON) following the manufacturer's instructions. Quantitative real-time PCR was performed using 2x qPCRBIO SyGreen 1-Step Lo-ROX (PCRBIO System, London, United Kingdom) using gene-specific primers (Table 1). The amplification cycle included 15 min of reverse transcription at 45 °C, 2 min of polymerase activation at 95 °C, 40 cycles of amplification for all genes with 5 s of denaturation at 95 °C, and 15 s of annealing at the temperature suitable for each gene.

2.6 Statistical Analysis

All data were expressed as mean ± SD and analyzed by GraphPad Prism software (GraphPad Prism Inc., San Diego, CA). Statistical significance was determined by a statistical threshold of $p < 0.05$.

3 Results

3.1 UCMSCs Exhibited MSC Phenotype

UCMSCs exhibited certain characteristics of mesenchymal stem cells, as suggested by the International Society for Cellular Therapy (ISCT). These included display of fibroblast-like shape when adhering on a plastic surface (Fig. 1a), successful differentiation into adipocytes which were positive with Oil Red dye staining (Fig. 1b), differentiation into osteoblasts which were positive with Alizarin Red (Fig. 2c), and differentiation into chondroblasts which were positive with Alcian Blue (Fig. 1d). They also displayed the MSC immunophenotype, including being positive for CD44 (99.02%), CD73 (95.05%), and CD90 (91.98%) (Fig. 1k–m) and being negative for HLA-DR (0.68%), CD14 (5.76%), and CD34 (5.15%) (Fig. 1h–j).

3.2 Adherence of UCMSCs on Treated and Non-treated Porous Scaffolds

Under microscopy, adhesion of UCMSCs increased on both the non-treated and treated scaffolds during culturing. On the non-treated scaffolds, UCMSCs attached and formed cellular clusters (Fig. 2a). The cellular clusters were gradually bigger and spread out on the 7th day (Fig. 2b). After 14 days of culture, the growth of UCMSCs increased and created links with structured fibers. The cell plaques were clearly observed (Fig. 2c). On day 21, the plaques were significantly larger than the previous days (Fig. 2d).

On the treated scaffolds, after 3 days of culture, UCMSCs attached on the scaffolds to form large cell plaques. These cell plaques grew rapidly and became larger on the 7th and 14th day (Fig. 2f–g), and the fibers of the scaffolds were completely linked together. On the 21st day, the cell plaques were observed to be thicker than the previous days (Fig. 2h).

UCMSCs (on scaffolds) which stained blue from nuclei staining with Hoechst 33342 stain

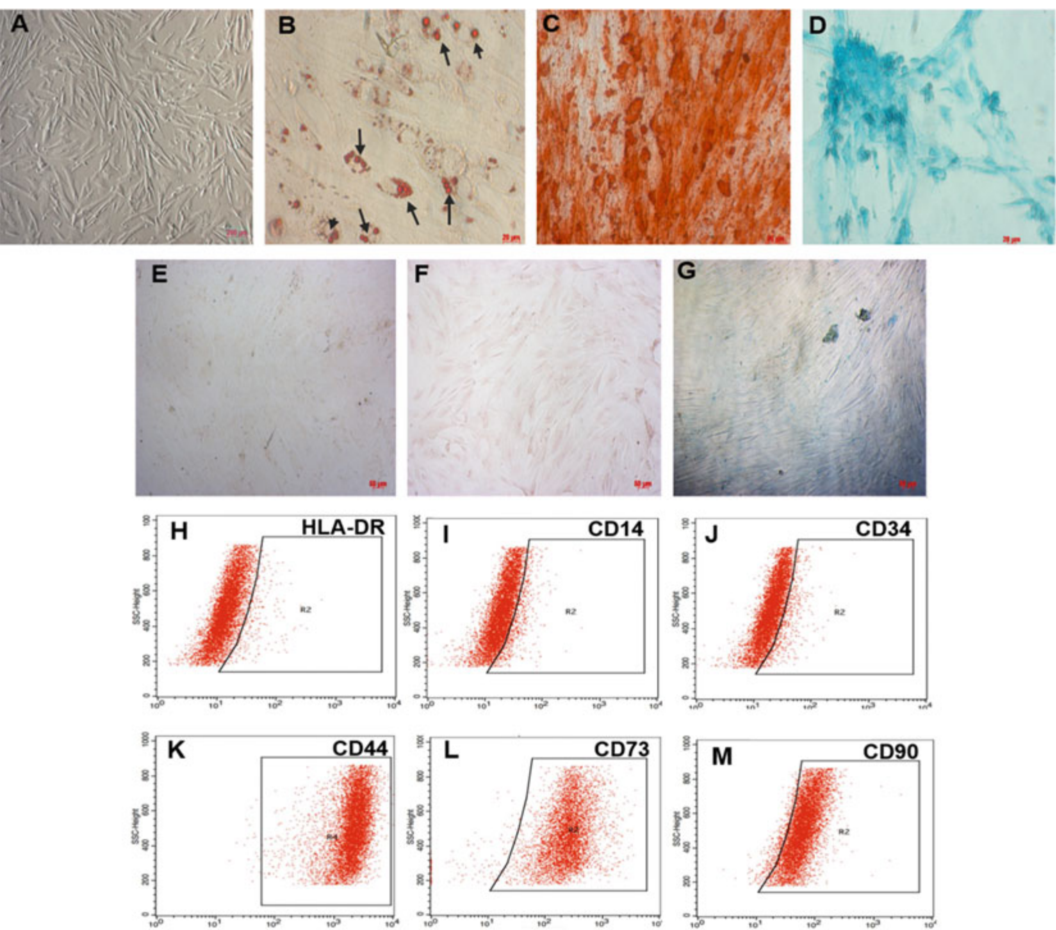


Fig. 1 UCMSC phenotype. UCMSCs express the characteristics of mesenchymal stem cells: fibroblast-like morphology (**a**), accumulation of lipids (**b**), extracellular calcium (**c**), and proteoglycans (**d**), as detected by Oil Red, Alizarin Red, and Alcian Blue after induction; the controls were negative with these dyes (**e, f, g**). The results of flow cytometry showed lack expression of HLA-DR (**h**), CD14 (**i**), and CD34 (**j**) and positive expression of CD44 (**k**), CD73 (**l**), and CD90 (**m**)

were observed via fluorescent microscopy (Fig. 3). The results showed that UCMSCs could attach on the surfaces and pores of scaffolds. It was observed that fewer cells attached on the non-treated scaffolds than on the treated scaffolds. This indicated that UCMSCs attached on the surface of the treated scaffolds to a greater extent. Cell counting showed that the number of cells that attached on the treated scaffolds was significantly higher than the non-treated scaffolds by 1.33 ± 0.12-fold ($p < 0.05$) after 7 days of culture (Fig. 4).

All above results demonstrated that the adhesion of UCMSCs on the treated scaffolds was better than on the non-treated scaffolds. Therefore, treated scaffolds were used in subsequent experiments.

3.3 UCMSC Proliferation on Pretreated Porous Scaffold

After 3 days of culture, UCMSCs attached on the pretreated scaffold. At a density of 10^5 cells/

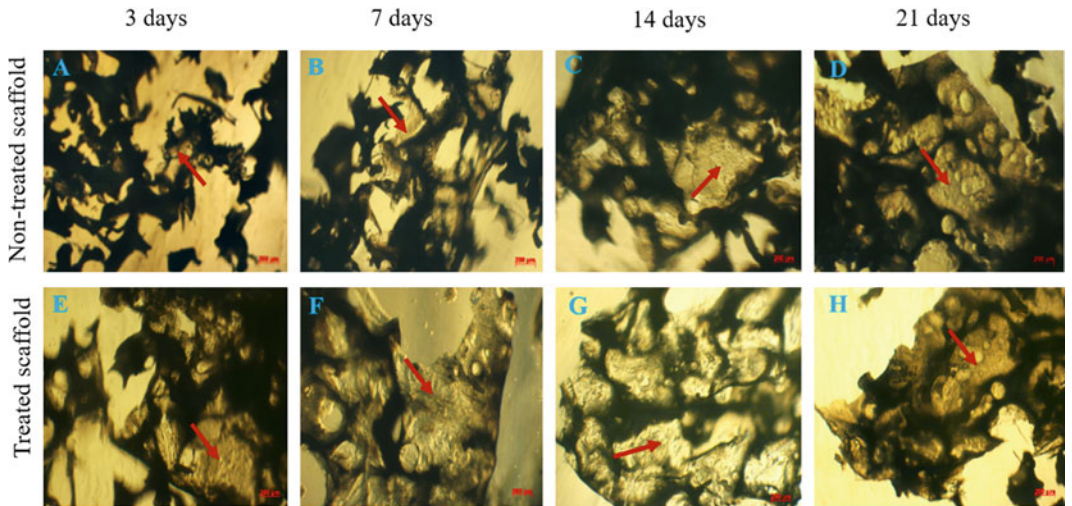


Fig. 2 The adhesion of UCMSCs on the Variotis scaffold. UCMSCs on non-treated scaffold after culture for 3 days (**a**), 7 days (**b**), 14 days (**c**), and 21 days (**d**); UCMSCs on treated scaffold after culture for 3 days (**e**), 7 days (**f**), 14 days (**g**), and 21 days (**H**)

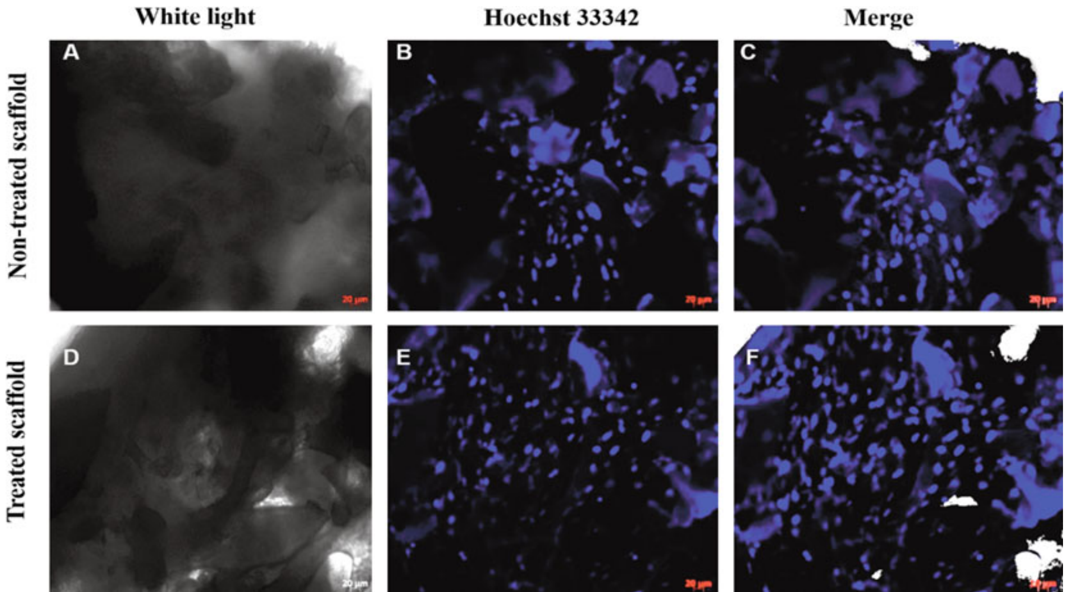


Fig. 3 Cell adhesion observed using Hoechst 33342 after culturing for 7 days. The non-treated scaffold with cells observed under white light (**a**), or fluorescent light (**b**), and the merged image of white light and fluorescent light (**c**). The treated scaffold with cells under white light (**d**), or fluorescent light (**e**), and the merged image of white light and fluorescent light (**f**)

0.005 g scaffold (Fig. 5a), the cells formed clusters, while at the density of 2×10^5 cells/ 0.005 g scaffold, the cells spread out to create small plaques (Fig. 5e). At the 7th day, these cells consecutively grew and filled out on all scaffolds (Fig. 5b, f). The cell plaques were bigger and distinctly defined after 14 and 21 days (Fig. 5c, d, g, h). Meanwhile, at a density of

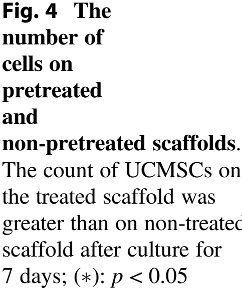
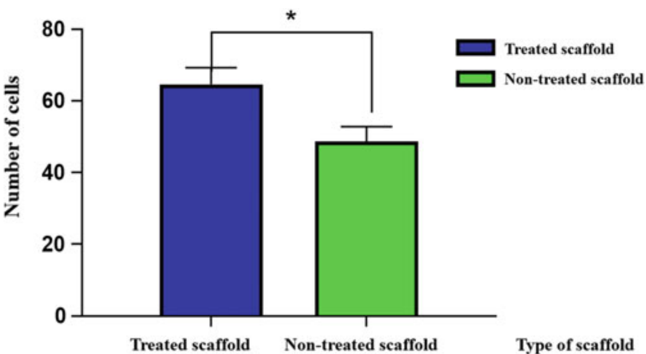


Fig. 4 **The number of cells on pretreated and non-pretreated scaffolds**. The count of UCMSCs on the treated scaffold was greater than on non-treated scaffold after culture for 7 days; (∗): $p < 0.05$



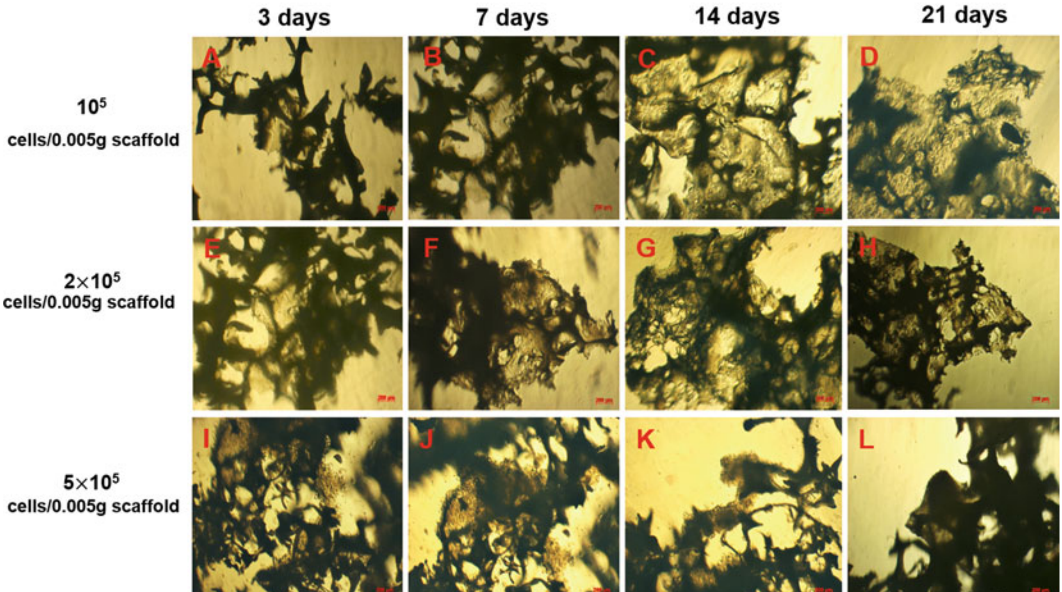


Fig. 5 **The cells were seeded on the scaffold at different densities.** Cells at a density of 10^5 cells/5 mg scaffold after culturing for 3 days (**a**), 7 days (**b**), 14 days (**c**), and 21 days (**d**). Cells at a density of 2×10^5 cells/5 mg scaffold after culturing for 3 days (**e**), 7 days (**f**), 14 days (**g**), and 21 days (**h**). Cells at a density of 5×10^5 cells/5 mg scaffold after culturing for 3 days (**i**), 7 days (**j**), 14 days (**k**), and 21 days (**l**)

5×10^5 cells/0.005 g scaffold, UCMSCs tended to cluster together before adhering on the scaffolds (Fig. 5i). The cell cluster was loosely connected and could not enter inside these scaffolds (Fig. 5j, k). At the 21st day, the clusters were no longer attached on the scaffolds and were removed after replacement with fresh medium (Fig. 5l).

To evaluate cell expansion on the scaffold, we used the MTT assay to measure cellular proliferation of the various seeded cell densities on the pretreated porous scaffold. It was generally noted that the OD index of MTT assay indicated growth of UCMSCs on the pretreated scaffolds after seeding up to day 14. However, the results in Fig. 6 showed that proliferation rates of UCMSCs of the various cell densities were different (Fig. 6).

At the 3rd day of culture, the proliferation rate of UCMSCs seeded at a density of 10^5 cells/

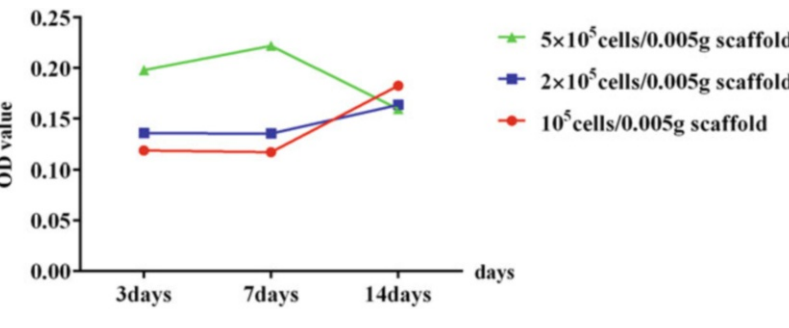


Fig. 6 Cell proliferation measured by MTT assay. UCMSCs slowly grew after 7 days of seeding with 1×10^5 and 2×10^5 cells/0.005 g scaffold, while strong proliferation was recorded for the density of 5×10^5 cells/0.005 g scaffold. However, cell growth was significantly reduced at day 14; on this day, the increase of cell proliferation was found for the other cell densities

0.005 g scaffold was slower than the other groups (2×10^5 and 5×10^5 cells/0.005 g scaffold). For 5×10^5 cells/0.005 g scaffold density, at the day 7th of culture, the proliferation rate was highest and was 1.13 ± 0.08-fold greater compared to that at day 3 ($p < 0.05$) for this group. The cell proliferation rate at day 14 was significantly reduced compared to day 7 and lower than that at day 3 of culture. For the other densities (10^5 and 2×10^5 cells/0.005 g scaffold), the proliferation seemed stable from day 3 to day 7 of culture. However, at the 14th day, these cells considerably expanded and achieved a higher concentration than that at day 7; there was a 1.33 ± 0.003-fold and a 1.13 ± 0.04-fold ($p < 0.05$) increase in the densities of 10^5/0.005 g scaffold and 2×10^5 cells/0.005 g scaffold, respectively. These results demonstrated that the density of 10^5 cells/0.005 g scaffold induced robust cell expansion on pretreated porous scaffolds after 14 days of culture. Thus, this density was used for subsequent experiments.

3.4 Osteogenic Differentiation of UCMSCs on the Scaffold

3.4.1 Change in Morphology

After 7 and 14 days of induction (in the differentiation medium) of 10^5 cells/0.005 g scaffold on pretreated porous scaffolds, the morphology of UCMSC population showed no negligible change (Fig. 7b, c) compared with those in the controls (no induction and culture in culture medium). However, at 21 days of differentiation, these cells condensed together and attached on the fibers of the scaffold (Fig. 7d). Condensation was clearly observed on the 28th day of induction (Fig. 7e), while UCMSCs consecutively spread and filled out the scaffold after 42 days of culturing (Fig. 7f).

3.4.2 Accumulation of Extracellular Ca^{2+}

After 21 and 28 days of osteogenic differentiation, cells on the scaffold became positive with Alizarin Red, and the color was stronger at day 28 (Fig. 8). However, the deposition of Ca^{2+} was not detected in the control sample (undifferentiated). The result of staining with Alizarin Red showed that there was an accumulation of extracellular calcium.

3.4.3 The Expression of Osteogenic Genes

During induction, the osteogenic genes changed upon investigation. *Runx2* was known as a transcriptional factor to activate osteogenesis. Our results showed that the expression of *runx2* was upregulated compared to control group (undifferentiated) (Fig. 9). After 7 days of induction, the expression of *runx2* was stronger and higher than non-induced cells by 2.5 ± 0.45-fold ($p < 0.05$).

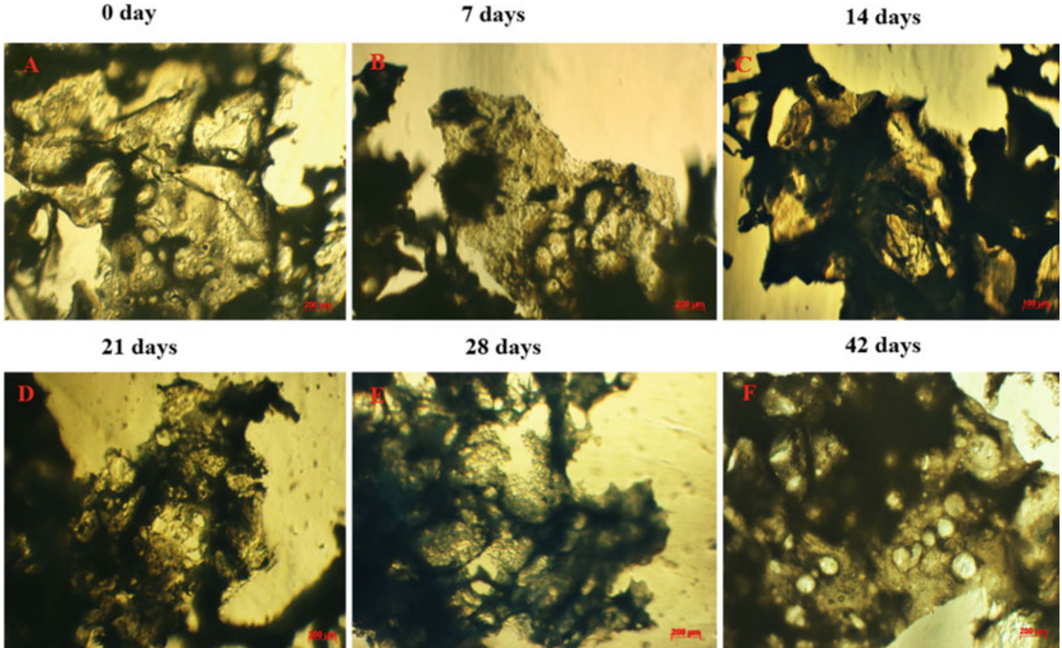


Fig. 7 **The shape of UCMSCs in the osteogenic medium**. Non-differentiated cells (**a**), differentiated on scaffold for 7 days (**b**), 14 days (**c**), 21 days (**d**), 28 days (**e**), and 42 days (**f**)

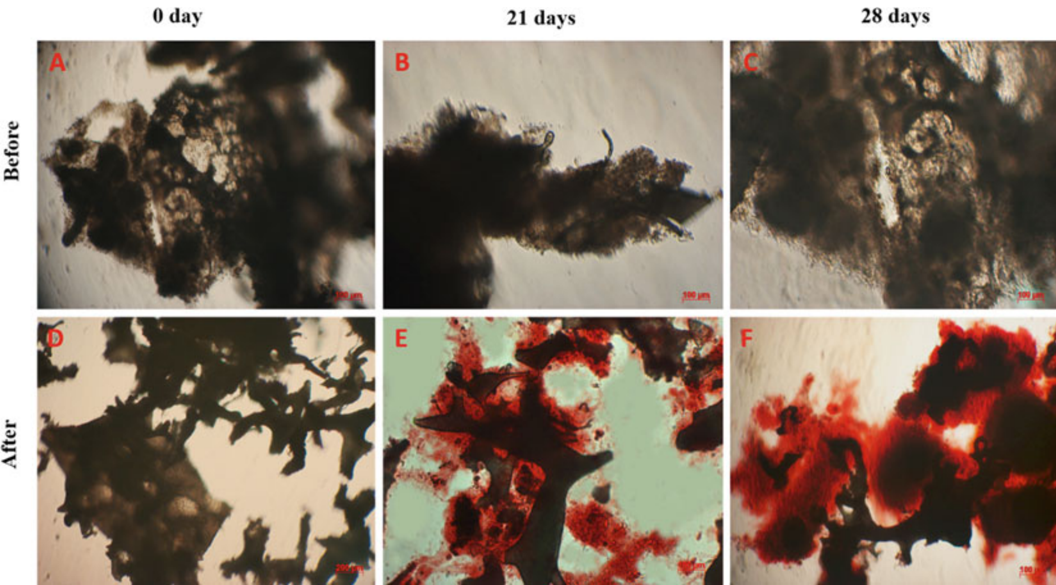


Fig. 8 **Results of Alizarin Red staining on UCMSCs**. Cells were cultured in normal growth medium (**a**), in osteogenic medium after 21 days (**b**), and for 28 days before dying (**c**). Cells were cultured in normal growth medium (**d**), or osteogenic medium after 21 days (**e**), or osteogenic medicine after 28 days (**f**)

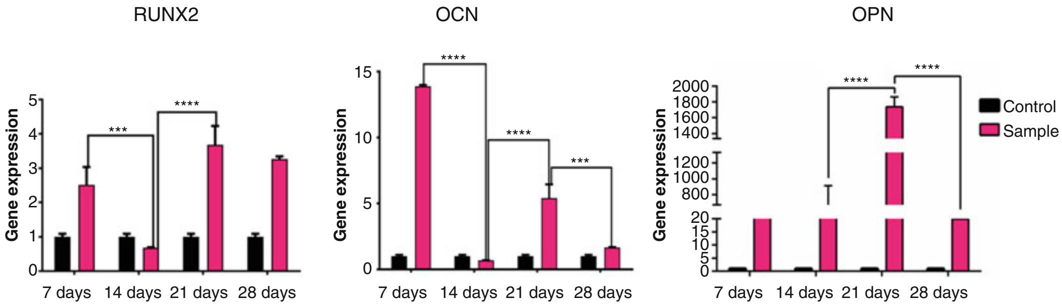


Fig. 9 Gene expression during osteogenic differentiation. Osteogenesis-associated genes, such as *runx2*, *OCN*, and *OPN*, were enhanced during induction, compared to the control group, particularly at the 21st day; (∗): $p < 0.05$

However, this expression significantly decreased at day 14 of differentiation and was lower than that for cells in the control group by 0.68 ± 0.06-fold and lower than them at the 7th day of differentiation by 3.7 ± 0.63-fold. On the 21st day, the expression of *runx2* in induced group was enhanced and reached the maximum and was higher than them in the 7th day by 1.46 ± 0.25-fold ($p < 0.05$). After 28 days of induction, *runx2* was negligibly decreased compared to the 21st culture day; however, that value was still higher than the control group by 3.26 ± 0.2-fold.

The expression profile of *runx2* correlated with *OCN* profile. The expression of *OCN* was upregulated to the highest level by 13.9 ± 1.69-fold compared to the control group on the 7th day of induction. On the 14th day, *OCN* expression sharply decreased compared to the 7th day and to control (20.2 ± 0.08-fold and 0.69 ± 0.06-fold, respectively). However, on the 21st day, the *OCN* expression increased by 7.85 ± 1.42-fold compared to the 14th day and was 5.4 ± 1.46-fold greater than the control group (non-induced). On the 28th day, the expression of *OCN* decreased and was lower by 3.24 ± 0.61-fold than that at day 21; meanwhile, the expression was higher than non-differentiated cells by 1.66 ± 0.14-fold.

Moreover, *OPN* gene expression increased from day 7 to day 21 and was higher than the control group. On the 7th day, the expression of OPN was stronger than that of the non-differentiated ($p < 0.05$). On the 14th day, the expression of *OPN* increased by 2.25 ± 0.65-fold compared with the 7th day and reached the highest level on the 21st day. However, the expression was considerably reduced after 28 days of induction.

4 Discussion

Bone tissues are widely used in bone grafting to treat bone diseases, trauma, and bone cancer. This study aimed to investigate the expansion and differentiation of UCMSCs on porous scaffolds to develop in vitro-engineered bone tissues for clinical applications.

In the first experiment, we expanded and characterized the UCMSCs. According to the International Society for Cellular Therapy (Dominici et al. 2006), human mesenchymal stem cells should be defined by adherence on culture surface, expression of specific markers (CD73, CD90, and CD105; lack of CD14, CD34, CD45 or CD11b, CD79 alpha or CD19, and HLA-DR), and differentiation capacity into adipocytes, osteoblasts, and chondrocytes in vitro. The results from our study showed that UCMSCs, after thawing, retained the characteristics of mesenchymal stem cells.

Indeed, in this study, we also compared the proliferation rate of UCMSCS on both pretreated and non-pretreated scaffolds. The results showed that the pretreated scaffold showed significant results compared to non-pretreated scaffold. The cell density for seeding on the scaffold was also evaluated at three different doses: 10^5, 2×10^5, and 5×10^5 per 0.005 g scaffold. In fact, the

proliferation, distribution, and differentiation of MSCs on scaffold could be affected by the cell seeding density (Zhou et al. 2011; Goldstein 2001). A previous study showed that cell-cell communication and paracrine signaling increased when cells were cultured at high density (Yassin et al. 2015).

DJ Warne et al. demonstrated that cell migration and growth were reduced by contact inhibitors in areas of high cell density. Thus, at a density of 5×10^5 cells/0.005 g scaffold, UCMSCs grew robustly during the first 7 days. However, on the 14th day of culture, the proliferation was greatly reduced and lower than that at the 3rd day. The number of cells was considerably increased from day 7 to day 14. The statistical results showed that the OD at day 14 of that density was not significantly different from the density of 5×10^5 cells/0.005 g scaffold at day 3. Therefore, at 10^5 cells and 2×10^5 cells/0.005 g scaffold densities, UCMSCs can continue to proliferate after 14 days. On the 14th day, the OD indexes of these densities were not considerable. The results suggested that the densities of 10^5cells/0.005 g scaffold could be considered as the appropriate density for further experiments.

In the next experiments, 10^5 UCMSCs/0.005 g scaffold were seeded on the pretreated scaffold after 14 days of expansion and were induced into osteoblasts in the osteogenic-inducing medium. After the 28th day of differentiation, UCMSCs clearly condensed to form clusters of cells found on the scaffold. Indeed, the condensation was considered as a prediction of early bone morphology. When mesenchymal cells differentiate into osteoblasts, these cells experience two main events, such as condensation and recruitment of other osteoblasts (Hall and Miyake 2000; Huycke et al. 2012). Initiation of the condensation was a result of three processes, including the enhancement of mitotic activity, aggregation, and failure of a cell growth at the center (Hall and Miyake 2000).

To confirm that these UCMSCs on the scaffolds were successfully differentiated to osteoblasts, these complexes of UCMSCs and scaffold were stained with Alizarin Red to detect the accumulation of calcium. The results confirmed that differentiated UCMSCs on the scaffold successfully accumulated calcium compared to the control (undifferentiated). Moreover, these differentiated UCMSCs expressed osteoblast-specific genes, including *runx2*, *OCN*, and *OPN*. During the osteogenic differentiation, *runx2* plays an important role in osteogenic differentiation of mesenchymal stem cells to the osteoblast and is express in the early stages of osteoblast differentiation (Jafary et al. 2017). Hence, the *runx2* expression has to be downregulated in the late stage of the osteogenic differentiation in the mature osteoblasts.

OCN is the most characteristic non-collagen protein of the osteoblasts, which expresses the cellular limitation of mineralized tissues, such as the bone extracellular matrix, the odontoblast cell, the cemented matrix, and cartilage cell hypertrophy (Sloan 2015). The expression of *OCN* was only found in the osteoblast (Wei and Karsenty 2015). Therefore, *OCN* is widely used as a cellular marker for osteoblast.

Moreover, the high expression of *OPN* was known as a marker of mature osteoblast. These genes were regulated by *runx2* gene via binding to the promoter of them (Ducy et al. 1997; Lian et al. 1998). In another study by Bruderer et al. (2014), the authors indicated that *runx2* helps to maintain the expression of *OPN* (Bruderer et al. 2014). This explains the relation of these genes in our research study. In the study by Huycke et al. (2012), the process of osteogenic differentiation included three steps: proliferation, transition, and maturation. In each step, the gene expression was different from each other. This was shown in our study and the research of Ding H et al. (2014). Notably, the low expression of *runx2* on day 14 was explanted by induction of the cells in the transition step (Huycke et al. 2012; Kong and Hinds 2012). When comparing it with the study of Huang et al. (2007), the enhancement of all genes demonstrated that UCMSCs could differentiate into mature osteoblasts on day 21 (Huang et al. 2007).

5 Conclusion

This study demonstrated that UCMSCs could adhere, proliferate, and differentiate on the porous scaffold to osteoblasts. At the density of 10^5cells/0.005 g of scaffold, the UCMSCs proliferated well on the scaffold (during day 1–14 of seeding). Furthermore, these cells at 14 days could be successfully differentiated into osteoblasts, which exhibited particular morphology and accumulated extracellular calcium expressed in the osteoblast-specific genes (*runx2*, *OPN*, and *OCN* after induction in the osteogenic medium for 21 days). These results suggested that in vitro-engineered bone tissue can be produced by UCMSCs and porous scaffold (Variotis scaffold).

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Competing Interests The authors declare that they have no conflicts of interest.

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