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REVIEW

Mesenchymal stem cell sheets: a new cell-based strategy for bone repair and regeneration

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Abstract Mesenchymal stem cells (MSCs), a class of adult stem cells, are considered a promising source for bone regeneration. Although combining MSCs with biomaterial scaffolds offers an interesting clinical strategy for bone tissue engineering, the presence of the scaffolds could induce an undesirable effect on cell–cell interactions. Moreover, before the application of scaffold materials in bone tissue reconstruction, cells must be manipulated with proteolytic enzymes, such as trypsin or dispase that degrade extracellular matrix (ECM) molecules and cell surface proteins, which can result in the cell damage and loss of cellular activity. Therefore, the development of alternative strategies for bone regeneration is required to solve these problems. Recently, a novel tissue engineering technology named 'cell sheet' has been efficaciously utilized in the regeneration of bone, corneal, cardiac, tracheal and periodontal ligament-like tissues. The cell sheet is a layer of cells, which contains intact ECM and cell surface proteins such as growth factor receptors, ion channels and cell-to-cell junction proteins. MSC sheets can be easily fabricated by layering the recovered cell sheets without any scaffolds or complicated manipulation. This review summarizes the current state of the literature regarding the use of MSCs to produce cell sheets and assesses their applicability in bone tissue regeneration and repair.

Keywords Bone regeneration · Bone tissue engineering · Cell sheets · Cell sheet technology · Mesenchymal stem cells (MSCs) · Scaffolds-free tissue engineering

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Introduction

Tissue engineering approaches for bone regeneration by seeding cells into a scaffold have recently produced promising clinical results (Gao et al. 2009). As a consequence, the use of scaffolds in combination with

osteogenic cells has become the gold standard in bone tissue engineering strategies (Pirraco et al. 2011). However, the usual method of tissue engineering involving the injection of isolated cell suspensions or the seeding of cells into biodegradable scaffolds may present some complications such as the cell loss, small quantities of seeded cells and inflammatory reactions with degradation of the scaffolds (Chen et al. 2016). In addition, this method has failed to produce the required results due to cell necrosis at the bulk of the scaffold related to nutrients diffusion and deficient oxygen (Pirraco et al. 2011). To overcome these limitations, the scaffold-free approach may be a suitable alternative to scaffold-based tissue engineering (Ma et al. 2010; Yorukoglu et al. 2017). In 2004, Nishida et al. established an innovative technique called 'cell sheet', in which cells were harvested together with endogenous extracellular matrix (ECM) and intact cell–cell contacts (Nishida et al. 2004). Cell sheet technology offers useful advantages with respect to cell suspension seeding strategy for bone tissue engineering. Most importantly, cell sheet and deposited ECM can be attached to host tissues with minimal loss of cells (Gao et al. 2009). To be specific, these cell sheets were formed from hyperconfluent cells until they produce extensive cell-to-cell interactions and synthesize a great quantity of ECM (Yorukoglu et al. 2017). In the last few years, cell sheets of mesenchymal stem cells (MSCs) have been widely utilized for the regeneration of many tissues and organs including bone, meniscus, cartilage, tendons, tooth, periodontal tissue and skin (see Table 1 for references). Moreover, MSC sheets have been also used for the regeneration of cornea (Gomes et al. 2010), cardiac tissue (Miyahara et al. 2006; Wang et al. 2007; Zhang et al. 2010; Huang et al. 2013; Haraguchi et al. 2014; Tano et al. 2014; Chang et al. 2015; Kawamura et al. 2015; Tanaka et al. 2016), nasal epithelium (Kavuzlu et al. 2017), blood vessels (Zhao et al. 2012), wound healing (McLaughlin and Marra 2013), digestive fistula (Rahmi et al. 2016), oral ulcers (Lee et al. 2017) and spinal cord defects (See et al. 2011; Okuda et al. 2017) (Table 1).

Cell sheets are thin films of about 80–150 μm thickness that can be generated in 2 weeks of In vitro culture using MSCs, as observed for example, using human PDL stem cells (PDLSCs) and human jaw bone mesenchymal stem cells (JBMSC) (Chen et al. 2007; Wang et al. 2016b).

Importantly, cell sheets contain a large amount of ECM proteins and cell-to-cell junctions, and therefore can be transplanted directly to tissue without the use of additional scaffolds (Iwata et al. 2015). This methodology preserves cell–cell interactions and the structure of ECM due to the fact that enzymatic digestion of the cells is not needed before application (Gao et al. 2009; Nakamura et al. 2010). The cell sheet consists in different layers of cells (usually 4–5) with approximately 80–150 μm thickness embedded in its self-secreted-ECM (Xie et al. 2015), in which cells maintain intact cell surface proteins such as cell–cell junctions, adhesion molecules, growth factor receptors and ion channels. The best characteristic of cell sheets is that the intact ECM at the bottom of the sheet permits direct adhesion of the sheet to the target organ. Therefore, the transplantation of the cell sheet does not require suturing, because it is obtained by the adhesion of the ECM at the bottom of the sheet to the target organ (Oka et al. 2018). More importantly, it is demonstrated that cell sheets transplanted remain at the transplant site for an extended period of time, with a higher graft survival rate with respect to that obtained with cell transplantation using isolated cell injections (Sekine et al. 2011; Oka et al. 2018). Therefore, due to the fact that ECM is present on the basal surface of the cell sheets, they can be easily transplanted directly to tissue beds or even overlapped, generating three-dimensional tissue-like structures (Chen et al. 2015).

Cell sheets can be generated by culturing MSCs at high confluence on dishes coated with a thermo-responsive polymer (Kwon et al. 2000; Long et al. 2014; Shang et al. 2017). At 37 °C, the dish surface is slightly hydrophobic and therefore cells can adhere to the dish and proliferate. When the temperature is lowered to 20 °C, the hydrophobic surface of the dishes reversibly changes to hydrophilic, determining the gradual detachment of a sheet of cells from the culture surface (Fig. 1) (Kwon et al. 2000; Long et al. 2014; Shang et al. 2017). These temperature-responsive culture surfaces possess several advantages with respect to the enzymatic harvesting of cells from culture dishes because the ECM components, cell-to-cell connections and adhesive proteins were conserved by this method. As a consequence, cell sheets could offer unique features that retain the microenvironment of the cells by avoiding enzymatic treatment and

Table 1 Studies that evaluated the use of MSC-sheets for the regeneration of tissue and organs

Type of tissues/ organs that was regenerated	MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/biological agents	Study type: in vitro or in vivo Animal model used	Effects	References
Bone	human-; canine-; porcine-; rat-; mouse-; leporine-; rabbit bone marrow stem cells (BMSCs) (Table 2)	PLGA meshes, allograft bone; β-tricalcium phosphate (β-TCP) disks; coral particles; poly-ε-caprolactone (PLC)/ β-tricalcium phosphate (β-TCP); chitosan (CS)/ hyaluronic acid (HA) nanoparticles (NPs); tubular coral scaffolds (Table 2)	In vitro and in vivo: (Further details of each study are provided in Table 2)	Repair of large segmental bone defects; new bone formation; complete bone union; stimulate the formation of new bone in critical-sized bone defects; bone regeneration in animal model (Further details of each study are provided in Table 2)	(Ouyang et al. 2006a; Chen et al. 2007; Gao et al. 2009; Zou et al. 2009; Akahane et al. 2010a, b; Nakamura et al. 2010; Qi et al. 2012; Geng et al. 2013; Long et al. 2014; Xie et al. 2015; Chen et al. 2016; Kim et al. 2016; Ueyama et al. 2016; Wang et al. 2016a; Kim et al. 2017; Shang et al. 2017; Wang et al. 2017)
Cord defects/ spinal cord defects	See et al. 201: Rabbit BMSCs Okuda et al. 2017: Rat BMSCs	silicone disc No scaffolds	In vitro. In vivo: Fischer 344 rats (completely transected SCI model in rats) with 2-mm-sized defect of the spinal cord at the T-8 level	Tissue-engineered intervertebral disc (IVD)-like assembly was able to regenerate an ECM structure and induce an increase in the Col II gene expression BMSC sheets are efficient in the treatment of SCI and permits autologous transplantation without the use of scaffolds	(See et al. 2011; Okuda et al. 2017)
Meniscus	Rabbit BMSCs	Poly-(lactic-co-glycolic acid) (PLGA)	In vivo: adult male Sprague–Dawley rats (rat massive meniscectomized model)	Transplantation of MSC sheets promote meniscus regeneration and inhibit the progression of osteoarthritis in knee joint In addition, MSC sheet transplantation reduced the degree of cartilage degeneration, osteophyte formation and subchondral sclerosis	(Qi et al. 2016)
Cartilage	Qi et al. 2014: Rabbit BMSCs; Cui et al. 2016: Rabbit BMSCs	poly-(lactic-co-glycolic acid) (PLGA). No Scaffolds; cartilage-derived morphogenetic protein 1 (CDMP1) transgenic cell sheets	In vivo: New Zealand white rabbits with osteochondral defects In vivo: New Zealand white rabbits (thyroid cartilage defect animal model)	Incorporation of MSC sheet to PLGA/MCSs enhances cartilage regeneration and integration between repair cartilage and the surrounding cartilage CDMP1-BMSC sheets have a good cartilage differentiation activity and can be successfully used to repair rabbit laryngeal cartilage defects	(Qi et al. 2014; Cui et al. 2016)
Tendons	Human BMSCs	Demineralized bone grafts or frozen tendon grafts and ascorbic acid	In vitro	MSC Sheets can differentiate into the osteochondral lineage. MSCs sheets adopted the characteristic spindle-shaped morphology of tenocyte-like cells	(Ouyang et al. 2006a)

Table 1 continued

Type of tissues/ organs that was regenerated	MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/biological agents	Study type: in vitro or in vivo Animal model used	Effects	References
Tooth	Minipig Periodontal ligament stem cell (PDLSCs) and dental pulp stem cells (DPSCs)	Sheet wrapping the hydroxyapatite (HA)\tricalcium phosphate scaffold (TCP)	In vivo: inbred miniature pigs (minipigs)	Allogeneic dental MSC-mediated bio-root regeneration is a practical approach for restoring adult tooth function in preclinical animal models	(Wei et al. 2013)
Periodontal tissue	Gao et al. 2015: human PDLSCs Wang et al. 2016b: Human PDL stem cells (PDLSCs) and jaw bone mesenchymal stem cell (JBMSCs)	Titanium nanotubes (Ti)/hydroxyapatite (HA) Platelet-rich fibrin scaffolds	In vitro and in vivo: 6-week-old immuno-compromised mice	Ti/cell sheets/HA complex were able to regenerate the PDL tissue without requiring extra soluble chemical cues In vitro PDLSC and JBMSC sheets exhibited higher expression levels of periodontal tissue-specific genes; calcification-related and adhesion-related genes compared to the corresponding cells In vivo: construct composed of PDLSC sheet/PRF/JBMSC sheet produced periodontal structures containing periodontal ligament tissue and bone-like tissues	(Gao et al. 2015; Wang et al. 2016b)
Skin	McLaughlin and Marra 2013: human adipose-derived stem cells (ASCs) Chen et al. 2017: Human MSCs (hMSC)	Autologous split thickness skin graft (STSG)	In vivo: full-thickness murine wound model (thymic mouse model) In vivo: Sprague–Dawley (SD) rats. hMSC cell sheets and pre-vascularized hMSC cell sheets were implanted in a rat full thickness skin wound model covered with an autologous STSG	ASC cell sheets by recruiting wound healing factors increase migration and proliferation Stem cell sheets through increased delivery of growth factors and enhanced cell survival are able to increase angiogenesis in the wound bed and have wide clinical implications in chronic and acute wound healing Pre-vascularization enhances therapeutic effects of human MSC Sheets in full thickness skin wound repair. Elevated presence of growth factors and cytokines in the pre-vascularized cell sheet exert a beneficial paracrine signaling during wound repair	(McLaughlin and Marra 2013; Chen et al. 2017)

Table 1 continued

Type of tissues/ organs that was regenerated	MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/biological agents	Study type: in vitro or in vivo Animal model used	Effects	References
Cornea	Immature dental pulp stem cells (hIDPSC)	Covered with deepithelialized human amniotic membrane (AM)	In vivo: New Zealand White rabbits with total limbal stem cell deficiency (LSCD)	The transplantation of hIDPSC sheet is efficacious in the reconstruction of corneal epithelium in an animal model of LSCD	(Gomes et al. 2010)
Cardiac tissue	Rabbit BM-MSCs	No scaffolds; VEGF	In vitro and in vivo	MSC sheets decrease the infarcted area and accelerate angiogenesis in the peri-infarcted area	(Miyahara et al. 2006; Wang et al. 2007; Zhang et al. 2010; Huang et al. 2013; Haraguchi et al. 2014; Tano et al. 2014; Chang et al. 2015; Kawamura et al. 2015; Tanaka et al. 2016)
Nasal epithelium	Rabbit adipose-derived stem cells (ADSCs)	Polyglycolic acid, applied with bacitracine cream and left to heal	In vivo: New Zealand white rabbits	ADSCs induce the healing of the injured maxillary sinus mucosa of the rabbits	(Kavuzlu et al. 2017)
Blood vessel	Rabbit BMSCs	A tissue-engineered vascular graft (TEVG) was fabricated by rolling the MSC sheet around a mandrel	In vivo: New Zealand white rabbits	Tissue-engineered vascular graft (TEVGs) are helpful for the revascularization in humans. The method allows the production of completely biological and living autologous vascular grafts without smooth muscle cells, endothelial cells and exogenous materials	(Zhao et al. 2012)
Digestive fistula	Human BMSCs	No Scaffolds (Scaffold-free)	In vivo: nude NMRI mice	Cell sheet grafting resulted in minimal clinical inflammation, improved fistula healing, reduced tissue fibrosis and enhanced microvasculature density	(Rahmi et al. 2016)
Oral ulcers	Rabbit ADSCs	No Scaffolds	In vivo: New Zealand white rabbits	Ulceration resolved before in the group treated with MSC sheets than in the control group. Examination revealed full-thickness mucosa healing and complete basal cell coverage in the group treated with MSC sheets with respect control group	(Lee et al. 2017)

BMSCs bone marrow stem cells (BMSCs)

retaining cell–cell junctions and their deposited ECM (Nakamura et al. 2010).

Importantly, it has been shown that the formation of MSC sheets can be also obtained by using other methods such as pH change-, magnetism-, electricity- and light-induced methods (Jiang et al. 2017; Yorukoglu et al. 2017). For example, Guillaume-Gentil et al. have shown that electro-chemically-induced pH lowering at the bio-interface is able to instigate cell sheet detachment by using poly(allylamine hydrochloride) and anionic poly(styrene sulfonate) polyelectrolytes (PAH/PSS) multilayer thin films as substrates for MSCs culture (Guillaume-Gentil et al. 2011). The advantages of this method are that recovered MSC sheets remain viable and maintain their differentiation capacity, whereas a disadvantage

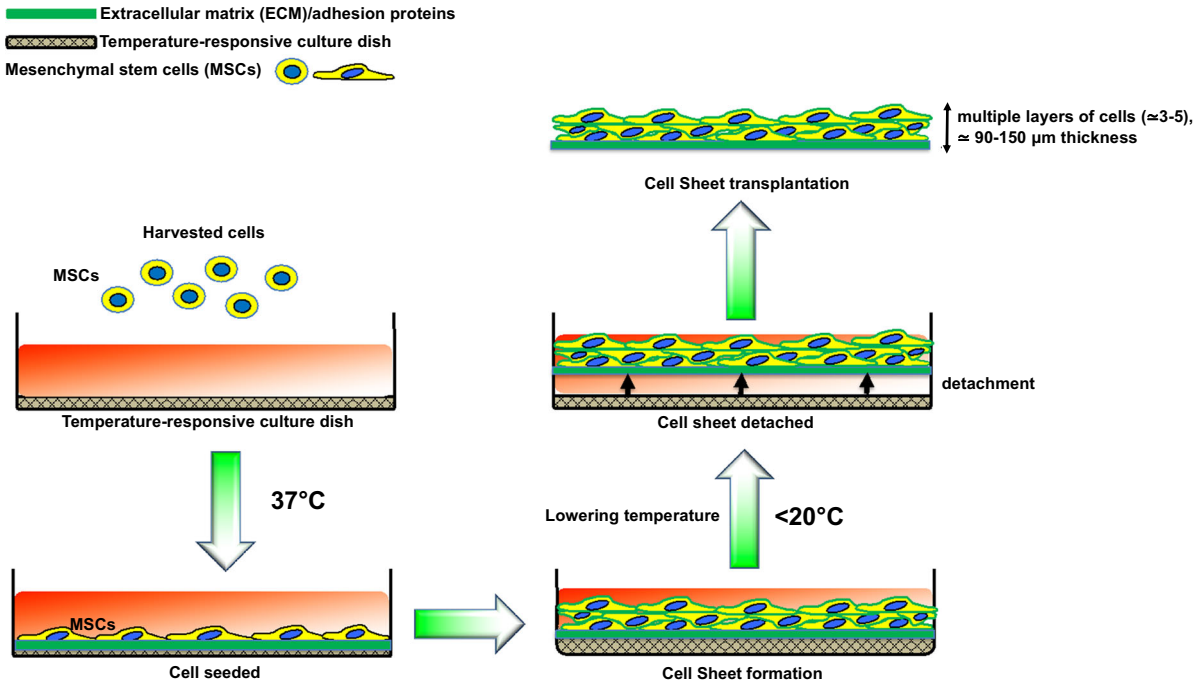

Fig. 1 Schematic representation of cell sheets formation

is that it requires cell cultures compatible with PAH/PSS substrates (Guillaume-Gentil et al. 2011). In the magnetic force-based tissue engineering technology, cells are incubated with magnetic nanoparticle-containing liposomes and a magnet is placed on the reverse side. In this condition, cells form multilayered cell sheets after 24-h of incubation (Ishii et al. 2014).

Another technique for engineering cellular tissues called electrochemical method has been proposed by Inaba et al. in 2009 (Inaba et al. 2009). In this method, cells are cultured on a self-assembled monolayer of alkanethiol. Then, the self-assembled monolayer can be reductively desorbed from the gold substrate by the application of a negative electrical potential, resulting in the detachment of cell sheets from the gold surface (Inaba et al. 2009). Furthermore, it was developed a light-induced cell sheet technology in which cell sheets can be detached from a TiO_2 nanodot-coated quartz substrate after UV365 illumination (Jiang et al. 2017). It is important to underline that the above described methods for cell sheet harvesting have taken advantage of surface property variations to induce cell detachment.

MSC sheets could be considered a promising tool for the regeneration of damaged tissues due to the fact that they do not require the use of non-self-materials such as biodegradable scaffolds, which can induce an inflammatory reaction in the host (Tsumanuma et al. 2011). Current treatment options to repair large bone defects caused by injuries, infections and degenerative diseases are mainly based on the usage of autologous or allogeneic bone grafts (Wen et al. 2017; Yorukoglu et al. 2017). Although these methods are commonly used, autologous bone grafts are limited by the availability of graft material and morbidity at the donor site such as pain or infections, whereas allogenic bone grafts are limited by disease transmission and potential immunological responses. Another current method for bone repair is the use of artificial bone, but it possesses several limitations such as weak synostosis, poor biodegradability and low mechanical strength (Wen et al. 2017). In this context, MSC sheet-based tissue engineering seems to be the appropriate strategy that can overcome the shortcomings of the previous methods. The aim of this review is to describe the cell sheets of MSCs and assess their applicability in bone tissue regeneration and repair (Table 2).

Use of MSC sheets for bone regeneration and repair

The use of MSC sheets for the regeneration of osteogenic tissue was firstly described by Ouyang

Table 2 Studies between 2006–2017 that assessed the use of MSC-sheets for bone repair and regeneration

MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/ biological agents	Study type: in vitro or in vivo animal model used	Effects	References
Human BMSCs	Demineralized bone grafts or frozen tendon grafts and ascorbic acid	In vitro	MSC Sheets can differentiate into the osteochondral lineage. MSCs sheets adopted the characteristic spindle-shaped morphology of tenocyte-like cells	(Ouyang et al. 2006a)
Porcine BMSCs	PLGA meshes Tube-like long bones were constructed by wrapping the cell sheet on to PLGA meshes	In vitro and in vivo Immunodeficient nude rats were used as subcutaneous transplant recipients	In vitro: formation of cartilage-like tissue during the 8-week culture period In vivo: dense mineralized tissue can be formed in subcutaneous sites by using PLGA meshes combined with BMSC sheets	(Chen et al. 2007)
Human BMSCs	Allograft bone MSC sheet wrapped onto structural allografts	In vivo: nude mice model (athymic nude mice) and rabbit model (New Zealand White rabbits). Implanted subcutaneously into nude mice as well as into the segmental radius defect of rabbits	The implantation of the MSC sheets enhanced the repopulation of bone grafts in nude mice. MSC sheets induced thicker cortical bone formation and more efficient graft-to-bone end fusion at the segmental bone defects in rabbits	(Zou et al. 2009)
Leporine BMSCs	Tubular coral scaffolds Cell sheets assembled with tubular coral scaffolds	In vivo*: nude* mice model	New bone formation with woven bone matrix subsequently maturing into fully mineralized compact bone	(Gao et al. 2009)
Rat BMSCs	No scaffolds	In vivo: rat femur nonunion model	Callus formation around the fracture site in the cell sheet- transplanted group. Bone union was obtained in the sheet group at 8 weeks. Femoral fracture was completely cured by the transplantation of a cell sheet	(Nakamura et al. 2010)
Rat BMSCs	β-tricalcium phosphate (β-TCP) disks	In vivo: Fisher 344 rats Porous β-tricalcium phosphate were transplanted subcutaneously into the backs of the rats. Immediately following implantation, the sheets were injected around the disks via a 16G needl	Calcification and bone tissue around the harvested disks when cell sheets were injected into the implanted disk immediately, calcification and bone tissue when cell sheets were injected into the implanted disks 1 week after disk implantation	(Akahane et al. 2010b)
Rat BMSCs	No scaffolds	In vivo: Fisher 344 rats	4 weeks after the subcutaneous injection, the injected areas showed hard mass formation. Each mass consisted of newly formed bone. Vascular network formation around the newly formed bone tissue at the subcutaneous injection site	(Akahane et al. 2010a)
Rabbit BMSCs	Recombinant human bone morphogenetic protein-2 (rhBMP-2)-loaded calcium sulfate (CS) combined with mesenchymal stem cell (MSC) sheets	In vitro and in vivo Rabbit model (New Zealand White rabbits)	In vitro: the alkaline phosphatase (ALP) of MSCs cultured on rhBMP-2-loaded CS was significantly higher than that of CS In vivo: the defects treated with MSC sheet-wrapped rhBMP-2-loaded CS showed significantly more bone formation than those treated with CS and rhBMP-2-loaded CS after implantation	(Qi et al. 2012)

Table 2 continued

MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/ biological agents	Study type: in vitro or in vivo animal model used	Effects	References
Rabbit BMSCs	Coral particles. The composite sheet was wrapped around a cylindrical mandrel to fabricate a tubular construct	In vitro and in vivo *Nude* mice model	In vitro: composite construct maintained its tubular shape and exhibited higher radiological density, compressive strength and greater extracellular matrix deposition In vivo: new bone formed ectopically on the composite constructs, composite sheets displayed radiological density similar to that of native bone	(Geng et al. 2013)
Mice BMSCs	Devitalized allografts Revitalized structural allografts wrapped with mesenchymal stem/ progenitor cell (MSC) sheets	In vitro and in vivo	In vitro: maintenance of the MSC phenotype in the sheets In vivo: MSC sheets stimulate cartilage formation at the graft-host junction and improved graft-host osteointegration and bone callus formation at 4- and 6-weeks post-surgery	(Long et al. 2014)
Rat BMSCs	Polyethylenimine–alginate (PEI–al) nanocomposites plus human BMP-2 complementary (c)DNA plasmid –	In vitro and in vivo *Wistar rats with calvarial defects*	In vitro: gene expressions of *SP7* and *ALP* in the PEI–al/pBMP-2 engineered cells sheets were significantly increased compared with those of the PEI–al/pEGFP In vivo: BMP-2-producing cell sheet group was efficient in promoting bone formation in the defect area	(Jin et al. 2014)
Human ethmoid sinus mesenchymal stem cells (hESMSCs)	Poly-sebacoyl diglyceride (PSeD) scaffold seeded with rBMSCs	In vivo: critical-sized calvarial defects in rats	Cell sheets of hESMSCs combined with porous PSeD scaffold seeded with rBMSCs remarkably promoted new bone regeneration in the repair of critical-sized calvarial defects of rat	(Xie et al. 2015)
Rat BMSCs	Stromal cell-derived factor-1 (SDF-1)	In vitro and in vivo Rat bone fracture model (rat tibia osteotomy model)	In vitro: elevated expression levels of bone morphogenetic protein 2, alkaline phosphatase, osteocalcin, and vascular endothelial growth factor in MSC sheets compared with MSCs cultured in medium In vivo: MSC sheet transplantation combined with the local injection of SDF-1 around the implantation site greatly promoted bone healing	(Chen et al. 2016)
Canine adipose-derived MSCs (Ad-MSCs)	Poly-ε-caprolactone (PLC)/β-tricalcium phosphate (β-TCP) PLC/β-TCP wrapped with osteogenic Ad-MSCs (OCS).	In vitro and in vivo Critical-sized bone defects in dogs (Beagle dog)	In vitro: ALP activity of OCS was significantly higher that that of undifferentiated Ad-MSCs. In addition, the mRNA expression levels of the osteo-specific genes ALP, RUNX-2 and BMP-7 were upregulated in OCS with respect to Ad-MSCs In vivo: OCS combined with PCL β/TCP stimulated new bone formation to repair critical-sized bone defects in dogs	(Kim et al. 2016)

Table 2 continued

MSCs type used for the generation of MSC-sheets	Combined with Scaffolds/ biological agents	Study type: in vitro or in vivo animal model used	Effects	References
Human BMSCs	Chitosan (CS)/hyaluronic acid (HA) nanoparticles (NPs) to deliver microRNA-21 (miR-21)	In vitro	miR-21 combined with hBMMSC sheets significantly enhanced the In vitro osteogenic differentiation of hBMMSC sheets in terms of upregulating calcification-related gene expression and enhancing alkaline phosphatase production, collagen secretion, and mineralized nodule formation	(Wang et al. 2016a)
Human BMSCs	Devitalized allograft segments	In vivo: Allogeneic bone grafts were obtained from mice of the 129/J strain for implantation into C57BL/6 J mice	MSC sheets showed more bony callus formed between allograft and host bone ends when compared to allograft alone. In addition, a significant increase of chondro-osteoclast activity was observed in the MSC sheet-grafted femur	(Shang et al. 2017)
Canine Adipose-Derived Mesenchymal Stem Cells (ADSCs)	Gelatin-induced osteogenic cell sheets (GCSs)	In vitro	Gelatin-induced osteogenic cell sheets (GCSs) were compared to conventional osteogenic cell sheets (OCSs). GCSs exhibited superior osteogenic transdifferentiation capabilities and a remarkable cell proliferation rate, compared to those of conventional OCSs	(Kim et al. 2017)
Rabbit BMSCs	Nanoscale hydroxy-apatite (nano-HA) and autologous platelet-rich fibrin (PRF)	In vivo: New Zealand white rabbits with critical-size defects (CSDs)	MSC sheets combined with nano-HA and granular PRF induce the bone regeneration in a rabbit calvarial CSD model	(Wang et al. 2017)

et al. in 2006 (Ouyang et al. 2006a). In their pioneering study, these authors analysed the effects of MSC sheets assembled onto demineralized bone grafts by using the wrapping method on the repair of large-bone and tendon defects. The results showed that when MSC sheets were assembled with large allografts, there was a formation of a periosteum tissue-like structure, which was important for the repair of bone defects (Ouyang et al. 2006a). Importantly, this study shed light on the fact that MSC sheets can be easily fabricated by layering the recovered cell sheets without any scaffolds or complicated manipulation and can be used as a novel strategy for clinical regeneration of large skeletal defects. Therefore, wrapping MSC sheets on devitalized allograft segments have been used as a tissue-engineered periosteum prior to transplantation. In this context, by comparing early-passaged young (P3) MSC sheets and later-passaged aged (P10) MSC sheets into a femoral allograft mouse model, it has been shown that young cultured MSC sheets can significantly increase the bone callus formation around allografts (Shang et al. 2017).

To evaluate if MSC sheets can be used for bone reconstruction, Chen et al. generated tube-like constructs composed of MSC sheets and polylactic–co-glycolic acid (PLGA) meshes and implanted them into nude rats (Chen et al. 2007). Their results showed that there was the formation of dense mineralized tissue in the subcutaneous sites of these rats. Importantly, three other studies have shown that it is possible to obtain viable bone constructs using MSC sheets obtained by similar wrapping methods (Zou et al. 2009; Long et al. 2014; Uchihara et al. 2015). In the first study, Zou and colleagues reported that when MSC sheets were wrapped onto an allograft and implanted in mice there was a formation of cortical bone (Zou et al. 2009). In the second study, Long et al. showed that allografts wrapped with MSC sheets were able to enhance graft-host osteointegration as well as bone callus formation (Long et al. 2014), whereas in the third study, Uchihara et al. co-transplanted cell sheets derived

from bone marrow stromal cells (BMSCs) with irradiated bone in the rat femur and demonstrated that MSC sheets can be used to facilitate osteogenesis of irradiated bones (Uchihara et al. 2015). Importantly, it has been shown that it is possible to obtain bone grafts in predetermined shapes and similar structure to the native bone using mineralised osteogenic BMSC sheets in combination with tubular coral scaffolds (Chen et al. 2007). Therefore, these findings also support the concept that the combination of MSC sheets with scaffolds is an encouraging technology for the regeneration of large bone grafts.

The strategy of using a combination of coral particles and MSC sheets for bone tissue engineering has also been successfully demonstrated by Geng et al. (Geng et al. 2013). Several other scaffolds have been combined with MSC sheets for bone regeneration, such as those composed of poly(sebacoyl diglyceride) (PSeD) (Xie et al. 2015) and poly-ε-caprolactone (PLC)/β-tricalcium phosphate (β-TCP) (Kim et al. 2016). PSeD scaffolds combined with human ethmoid sinus mucosa membrane (hESMSCs) and PLC/β-TCP scaffolds were able to stimulate the formation of new bone in critical-sized calvarial defects of rats (Xie et al. 2015; Kim et al. 2016).

Interestingly, to enhance osteogenesis of MSC sheets, they have also been combined with scaffolds containing biological agents (Table 2). In this context, bone morphogenetic protein-2-loaded calcium sulfate (Qi et al. 2012), chitosan/hyaluronic acid nanoparticles plus microRNA-21 (Wang et al. 2016a), nanoscale hydroxy-apatite combined with autologous platelet-rich fibrin (Wang et al. 2017) and polyethylenimine–alginate nanocomposites plus human BMP-2 (Jin et al. 2014) were successfully used to induce bone regeneration when combined with MSC sheets. Similar results were obtained by combining MSC sheets with biological agents and without scaffolds such as gelatin (Kim et al. 2017) and stromal cell-derived factor-1 (SDF-1) (Chen et al. 2016) (Table 2). For example, it has been shown that the supplementation of gelatin in osteogenic medium (GCSs) induces higher osteogenic differentiation abilities than conventional osteogenic cell sheets (OSCs) (Kim et al. 2017), whereas the addition of SDF-1 to MSC sheets was able to induce new bone formation in fractures and also bone union (Chen et al. 2016).

Although the use of MSC sheets in combination with bone grafts or scaffolds/biological agents have shown promising results, three other studies have shown that MSC sheets can be used also without any graft or scaffolds (Akahane et al. 2008, 2010a; Nakamura et al. 2010). Akahane et al. in 2008 were the first authors that investigated the graft-free use of MSC sheets to obtain bone tissue (Akahane et al. 2008). They generated rat MSC sheets using osteogenic supplements and rolled them to obtain tube-like structures that were subsequently transplanted into subcutaneous sites of rats. There was a formation of ectopic calcification 6 weeks after sheet transplantation, indicating that osteogenic MSC sheets can be used as osteogenic implants for bone tissue reconstruction (Akahane et al. 2008). Later, the same authors injected MSC sheets derived from BMSCs through a needle into subcutaneous sites and dead bone of rats (Akahane et al. 2010a). Their results confirmed that MSC sheets can be also transplanted via a needle without the use of graft or scaffolds to reconstruct hard tissue in osteonecrosis and non-union treatment. Currently, the possibility to use scaffold-free MSC sheets represents an intriguing strategy with several advantages over scaffold-based strategies, such as the absence of inflammatory reactions after transplantation to the host. These findings were confirmed by two different studies by Nakamura et al. and Ma et al, in which the use of MSC sheets without exogenous scaffolds showed that a femoral fracture was completely cured in a rat non-union model (Nakamura et al. 2010) and that BMSC sheets can accelerate the development of functional 3D bone tissues (Ma et al. 2010).

Limitations of cell-sheet technology

While there have been significant advances in the production of MSC sheets for bone regeneration, more than one parameter has been used such as the composition of the culture medium, the In vitro culture period and the use of cell sheets with or without scaffolds/grafts (Yorukoglu et al. 2017). Therefore, several questions are required to be solved before this technology goes into clinical practice. Moreover, the immunogenicity and the survival of MSC sheets after implantation should be investigated (Ouyang et al. 2006b). For this purpose, the autologous cell source should be used to avoid immunological rejection. Importantly, additional studies are still needed to

understand the choice of scaffold material and ideal culture conditions (Chen et al. 2007), and to determine any clinical applications of MSC sheet technology (Akahane et al. 2010b).

One limitation of the cell sheet technology is the possible risk of using ex vivo expanded MSCs. In fact, the growth of MSCs requires culture medium supplemented with fetal bovine serum (FBS), which can increase the risk of contamination and induce unfavourable cellular modifications. To replace the FBS and to reduce the possible re-implantation problems of cultivated MSC sheets, some investigators have used platelet-rich fibrin (PRF) as a possible substitute (Wang et al. 2017). Another important problem of the cell sheet technology is the limited blood supply that is necessary for bone regeneration (Yorukoglu et al. 2017). In fact, large and thick tissue grafts, such as those generated by the cell sheet technology, could induce the generation of necrotic cores due to limited passive diffusion of molecules such as nutrients and oxygen. In order to resolve this problem, several researchers are trying to produce 3D vascularized tissues using cell sheet technology (65).

Importantly, today, cell sheet manufacture is manual and requires a highly skilled operator to produce the sheets. Therefore, a number of questions have to be answered before this technique goes to clinical practice. First of all, a method of quality control for cell sheets is needed. For example, prior to the scheduled day of transplantation, the prepared autologous cell sheets should be subjected to various quality tests such as cell number, the percentage of cell viability, cell purity and thicknesses of cell sheets. Moreover, also unknown variation in the surfaces of materials could have a significant impact on safety, efficacy and consistency of a product (Kirby et al. 2018). Importantly, it is also essential to underline that in clinical applications, cell sheets should be also negative for Mycoplasma pneumoniae, endotoxin, bacteria, fungi and viruses (Yamamoto et al. 2017). Therefore, more extensive research and clinical trials are needed to define a method of quality control for cell sheets in clinical applications.

Conclusions and future perspectives

In the first studies, several biomaterials were combined with MSC sheets for the regeneration of the osteogenic tissues such as polylactic-co-glycolic acid (PLGA) meshes (2), coral particles (Geng et al. 2013), β-tricalcium phosphate (Akahane et al. 2010b; Kim et al. 2016), hydroxyapatite particles (Wang et al. 2016a), surface-modified titanium and zirconia (Zhou et al. 2010), coumarin-like derivative osthole (Gao et al. 2013) and a complex of polyethylenimine-alginate nanocomposites (Jin et al. 2014). Moreover, biological agents such as Notch activation by Jagged1 (Tian et al. 2017), vitamin C (Guo et al. 2015), stromal cell-derived factor-1 (Chen et al. 2016), platelet-rich fibrin (Wang et al. 2017) and simvastatin (Qi et al. 2013) were utilized in combination with MSC sheets to improve their osteogenesis. However, due to the fact that cell sheet engineering can improve the concentration of MSCs at the site of delivery without the use of external scaffolds, several studies have shown that is possible to use MSC sheets without any graft or scaffolds in bone regeneration and repair (Akahane et al. 2008, 2010b; Nakamura et al. 2010). Therefore, tissue-derived MSC sheets can be used either in scaffold/graft-free applications or in combination with various grafts or scaffolds to shorten the treatment period of bone regeneration. The principal drawback of the cell sheet technology is the potential induction of necrosis within the cell sheets caused by the absence of vascularization. Therefore, the current literature on this field encourages the production of 3D vascularized tissues by cell sheet engineering (Sakaguchi et al. 2015). Even if the major part of the studies used MSCs from human or animal bone marrow (BMSCs) to generate sheets for bone regeneration (Ouyang et al. 2006b; Chen et al. 2007; Gao et al. 2009; Zou et al. 2009; Akahane et al. 2010a, b; Nakamura et al. 2010), human ethmoid sinus mucosa membrane (hESMSCs) (Xie et al. 2015) and adipose-derived MSCs (Ad-MSCs) (Kim et al. 2016, 2017) have also been successfully used for this purpose.

To summarize, this review focused on a novel concept, which is the use of cell sheet technology to enhance the transplant efficiency and the bone regeneration ability of MSCs. Cell sheets not only offer an optimal microenvironment to enhance osteogenic differentiation potential of MSCs but also could be used as a promising approach for bone tissue regeneration and repair.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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