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Mesenchymal stem cells for vascular regeneration

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Abstract

Mesenchymal stem cells (MSCs) have tremendous potential for regenerative medicine, and have been researched for the treatment of cardiovascular diseases. MSCs are a promising cell type because of their ease of isolation and expansion, their multipotency and their low immunogenicity. However, in order to fully utilize the therapeutic potential of MSCs, it is important to understand the intrinsic property of MSCs and the role of the microenvironment in modulating MSC behavior and function. Microenvironmental factors such as mechanical cues, soluble factors and matrix properties not only regulate MSC differentiation, but also modulate MSC signaling to the surrounding environment. Understanding the properties of MSCs and the role of the microenvironment will be beneficial for developing *in vivo* therapies for the construction of tissue-engineered vascular grafts and the treatment of ischemic cardiac tissues.

Keywords

angiogenesis; bone marrow; extracellular matrix; mechanical strain; mesenchymal stem cell; myocardial infarction; shear stress; tissue engineering; vascular graft

In the USA, cardiovascular disease is a serious cause of mortality and morbidity. Three-quarters of all cardiovascular-related deaths are attributed to atherosclerosis and the resulting arterial clogging, myocardial infarction (MI) and stroke [1]. In 2005, it was reported that 469,000 coronary artery bypass procedures and over one million percutaneous interventions were performed [1]. Despite the improvement in overall quality of life using existing treatments, there is still a prevalent need for new treatments that can enhance blood perfusion to ischemic tissues, improve the patency of small-diameter bypass grafts and provide alternative options when there is no suitable autologous artery or vein for bypass grafting.

With the advent of technological advances in stem cell therapeutics and vascular tissue engineering, the ability to regenerate the vasculature may be one step closer to reality. Stem cells have the unique ability to give rise to progeny with more specialized cell function, as well as those that retain their stem cell state. Among the various types of stem cells, mesenchymal stem cells (MSCs) are a promising cell source because of their high expansion ratio, ease of

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isolation, ability to differentiate into vascular cell types and release proangiogenic factors and low immunogenicity. However, in order to develop novel therapies to restore dys-functional vasculature, it is necessary to understand how the microenvironment may affect MSC differentiation, signaling and organization into functional units and tissues for vascular therapy. As illustrated in Figure 1, the microenvironment includes chemical factors, such as soluble signaling factors, physical cues, such as matrix topography and matrix rigidity, and mechanical stimulation, in the form of shear stress and strain [2].

In this article, we will discuss the role of the microenvironment in modulating MSC phenotype and the potential of MSCs for vascular regeneration, with emphasis on the engineering of vascular grafts and neovasculature formation for treatment of MI.

Characterization of MSCs

Interest in MSCs began over 130 years ago when Cohnheim, a German pathologist, suggested that the bone marrow gave rise to fibroblast-like cells during the repair process [3,4]. Later in the 1970s, Friedenstein and colleagues demonstrated that whole bone marrow contained a heterogeneous population of adherent cells that could later generate bone and cartilage deposits [5]. These results were later confirmed and further investigated by several groups of pioneers in the field, who demonstrated that the adherent cells were multipotent and could differentiate towards a variety of lineages, including cardiovascular cell types [6–10]. Owing to the various definitions of MSCs used throughout the literature, the working definition of MSCs that will be used throughout this article is a class of adherent cells with spindle-shaped morphology, self-renewal capacity and ability to give rise to daughter cells with more specialized function.

MSCs can be found in numerous organs, including the bone marrow, fat, blood, liver and spleen [2,11–13], with bone marrow being the most characterized origin. Within the bone marrow, MSCs are adherent cells that contribute to the niche of non-adherent hematopoietic stem cells (HSCs). Although MSCs occupy only 0.01% of the total population of nucleated cells in bone marrow, *in vitro* they have a high expansion ratio of over 1 million-fold, while maintaining multi-lineage differentiation capacity [8,14]. They can be routinely expanded for over ten passages in media containing defined components (i.e., StemPro® MSC Serum Free Medium, Invitrogen, Carlsbad, CA, USA) or pre-screened fetal bovine serum.

Techniques to purify MSCs include Percoll® gradient centrifugation, selection by the adherent mononuclear cell population on tissue culture-treated Petri dishes or by immunophenotyping. There is no specific marker that can identify MSCs from other cell types, but MSCs generally express markers including STRO-1 (a stromal cell surface antigen), CD29 (integrin β 1), CD44 (receptor for hyaluronic acid and matrix proteins), CD105 (endoglin) and CD166 (cell adhesion molecule). On the other hand, they lack the expression of CD14 (monocyte surface antigen), CD34 (HSC surface antigen) and CD45 (leukocyte surface antigen) [15,16]. In some cases, such cell surface markers have been empirically used to isolate MSCs by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting. However, this surface marker profile is not standardized for all MSCs owing to the heterogeneity of cell phenotype from various origins and species. For example, CD34 appears to be expressed in MSCs derived from mice, but are absent in human MSCs [17]. Nevertheless, these surface markers are commonly used for characterizing MSCs.

MSC differentiation towards vascular phenotypes can be distinguished by the expression of experimentally identified specific markers or by functional assays. For smooth muscle cell (SMC) differentiation, markers specific to this cell type include cytoskeletal proteins α -smooth muscle actin (SMA), smooth muscle myosin, SM22 and calponin [18]. On the other hand, endothelial cell (EC)-specific markers include platelet-endothelial cell adhesion molecule 1 (CD31), vascular endothelial (VE)-cadherin and EC-synthesized glycoprotein von Willibrand

Factor (vWF). Functionally, ECs are also characterized by their ability to form tube-like structures in matrigel and uptake acetylated low-density lipoproteins.

As a result of various tissue sources for the generation of MSCs, differences may exist in terms of their growth characteristics, gene expression and differentiation potential. With respect to expansion rates, one study compared the growth curves between Ficoll-gradient centrifugation-derived human MSCs from the umbilical cord, bone marrow and adipose tissue [19]. The authors showed that the frequency of colony formation was highest in cells derived from adipose tissue and lowest in those derived from umbilical cord. On the other hand, umbilical cord-derived MSCs appeared to have the highest proliferation capacity, whereas the bone marrow-derived cells had the lowest capacity. Another study examining the gene-expression profiles of human bone marrow-derived MSCs reported that the top 36 of 50 most expressed tags by these cells were also expressed in umbilical cord-derived MSCs [20]. Although these two origins of MSCs shared similar immunophenotypes, the bone marrow-derived MSCs had higher expression of markers associated with osteogenesis, namely biglycan, *TSC22*, *CD44* and vitronectin. On the other hand, umbilical cord-derived MSCs appeared to express higher levels of genes associated with angiogenesis mediated by IL-1 and TNF- α pathways, including *CXCL6* (*GCP-2*), *IL-8* (or *CXCL8*), *IL1RL1LG* and *MMPI* (interstitial collagenase). These data implicate potential differences in the molecular signature and growth patterns of MSCs from various origins. However, it is unclear how these differences may modulate their differentiation capacity towards vascular lineages.

The advantages of MSCs as a cell source for vascular regeneration include the ease and accessibility of MSCs by a bone marrow aspiration in the iliac crest or lipoaspiration from liposuction procedures, high expansion potential and reproducible phenotypic characterization by FACS analysis [8].

One unique advantage of MSCs is their potential for allogenic cell delivery in immunocompetent patients. Their immune-privileged characteristic is partially due to the lack of expression of major histocompatibility complex (MHC) II antigens that are responsible for immune rejection, although MHC II expression could be induced by IFN- γ stimulation [21]. In addition, MSCs lack the expression of co-stimulatory molecules that activate T cells, including *CD40*, *CD80* and *CD86* [22]. MSCs have immunomodulatory effects of inhibiting the proliferation of T cells and B cells, and suppressing the function and maturation of dendritic cells [23–25]. These effects are mediated by both cell–cell contact and by the release of soluble factors that include TGF- β 1, HGF, IL-10 and prostaglandin E-2 [23,26,27]. Since MSCs do not appear to acquire MHC II cell surface antigens during adipogenic, chondrogenic and osteogenic differentiation, it is possible that MHC II antigens would not be expressed upon cardiovascular differentiation [21].

Soluble chemical factors & MSCs

Soluble chemical factors are multifaceted in their ability to modulate cell behavior and function (Table 1). In particular, they can signal vascular cells and MSCs to grow, proliferate, migrate, differentiate and modulate cellular release of chemical factors. The factors that influence MSCs include VEGF, PDGF, basic FGF (bFGF) and TGF- β . Conversely, MSCs can also release some of these growth factors to facilitate tissue regeneration in the microenvironment (Table 2).

VEGF-A is commonly produced by vascular and tumor cells, and has been shown to be expressed by MSCs as well. The release of VEGF from MSCs can promote the recruitment of ECs for angiogenesis in ischemic tissue and endothelialization in injured arteries. VEGF-A homodimers usually bind to VEGF receptor 1 (flt) or receptor 2 (fl k-1). However, recent studies show that MSCs do not express VEGF receptors, but VEGF can promote MSC proliferation and migration by activating PDGF receptors in MSCs [28]. In addition, PDGF,

bFGF and TGF- β are two other angiogenic growth factors that modulate MSC activity. As shown in Table 1, these factors play a role in inducing cell migration, proliferation and differentiation into vascular cell types. For example, VEGF, PDGF and bFGF appear to stimulate MSC migration [28–31]. VEGF and PDGF can induce MSC differentiation into an endothelial phenotype [29,32], whereas TGF- β signals SMC specification [33–36]. Proteomic profiling analysis revealed that TGF- β decreased the expression of gelsolin to promote the assembly of contractile filaments [33]. Furthermore, transactivation of genes could also induce some similar effects as soluble factors, as shown by the effect of MSC proliferation after adenoviral transduction of VEGF [29]. Together, these results indicate that soluble factors can modulate MSC phenotype.

Role of mechanical factors

Mechanical stimulation plays an important role in maintaining the phenotype of vascular cells. The endothelium is continuously subjected to fluid shear stress caused by blood flowing across this lining, and both the endothelial and smooth muscle layers experience cyclic strain resulting from pulsatile stresses from the beating heart. The role of mechanical factors on vascular cell behavior and phenotype has been well-documented [37–41], but the ability of mechanical stimulation to induce phenotypic changes in MSCs towards vascular lineages has only recently been investigated. Among the various forms of mechanical stimulation, shear stress and strain models are commonly studied.

■ Role of shear stress on MSCs

Under physiological conditions, the endothelium in arteries experiences shear stress within the range of 10 to 20 dynes/cm² [42,43]. The shear stress that results from blood flow appears to modulate EC function by activating downstream signaling pathways. To study the effect of shear stress on cell behavior, both *in vitro* and *in vivo* systems can be used. The advantage of the *in vitro* system is the ability to control the magnitude and direction of shear stress, whereas *in vivo* models are a step closer to translating *in vitro* findings to clinically relevant diseases [44]. Fluid shear stress is commonly modeled *in vitro* by laminar flow using a parallel-plate chamber as a 2D culture model, or in the case of interstitial flow, by a porous 3D matrix.

To date, studies of shear stress on MSC migration, proliferation and vascular differentiation remains limited in number. Table 3 summarizes the results from published findings related to shear stress effects on MSC phenotype and behavior. In one study using STRO-1-expressing rat bone marrow stromal cells, 36-h shear stress (~ 14 dyn/cm²) stimulation by pulsatile flow increased the number of smooth muscle myosin heavy chain-expressing cells, although SMA protein levels was not significantly different when compared with the static control group [45]. In another study, shear stress applied to murine C3H/10T1/2 embryonic mesenchymal progenitor cells led to induced expression of endothelial markers such as CD31, vWF and VE-cadherin at the mRNA and protein level after 12 h of steady shear stress levels of 15 dyn/cm² [46]. Shear stress could also stimulate endothelial function, including the uptake of acetylated low-density lipoproteins and formation of capillary-like structures on matrigel.

■ Role of mechanical stretch on MSCs

Physiologically relevant levels of strain in blood vessels range from 5 to 30% strain and 30 to 90 cycles per min [37]. In 2D culture, strain is often studied by uniaxial or equiaxial models in which cells are cultured on elastic substrates such as poly(dimethylsiloxane) (PDMS), which can be repetitively stretched to controllable strain magnitudes and frequencies. For 3D stretch models mimicking vascular environments, equiaxial stretch conditions can be simulated by embedding cells in a tubular matrix and then stretching the construct from the luminal side.

The effect of strain is well-characterized in vascular cell types, but not for MSCs [47]. As summarized in Table 3, we have previously shown that for human bone marrow-derived CD105- and CD166-expressing MSCs, 5% cyclic equiaxial strain at 60 cycles per min for 24 h downregulates SMA and SM22 α , whereas cyclic uniaxial strain transiently increases the expression of these markers [48]. However, in another study in which rat bone marrow-derived adherent cells were exposed to 10% cyclic uniaxial strain at 60 cycles per min for 7 days, the expression of SMA and h1-calponin was induced [49]. The seemingly opposing results may be attributed to differences in stimulation time, species-specific response or stem cell phenotype. In the former report, immunophenotypically characterized human cells were studied for a period of 24 h at 5% equiaxial strain, whereas the latter report utilized rat adherent cells without immunophenotypic characterization and examined the effect of 10% cyclic uniaxial strain for 7 days. Consequently, it is unclear which specific factors may be attributed to these conflicting results.

In addition to global gene-expression changes, uniaxial strain induces cell realignment in the perpendicular direction, relative to the direction of stretch. To assess whether uniaxial strain could promote MSC differentiation if cell orientation was restricted, we cultured human bone marrow-derived CD105- and CD166-expressing MSCs on microgrooves in the directions parallel or perpendicular to 5% uniaxial strain. Notably, global changes in MSCs were observed only when MSCs were kept aligned in the direction of uniaxial strain [50]. DNA microarray analysis showed an upregulation of h1-calponin expression, alterations in cell signaling and increases in cell proliferation. Together, these studies suggest that physiological levels of stretch can modulate MSC gene expression, cell alignment, proliferation and phenotype towards those that are consistent with smooth muscle characteristics.

■ Role of multimodal mechanical stimulation on MSCs

To mimic the dynamic physiological environment, multimodalities of mechanical stimulation have been studied in well-controlled *in vitro* environments. O'Cearbhaill *et al.* cultured bone marrow-derived CD73- and CD105-expressing human MSCs in the presence of pulsatile pressure (40–120 mmHg), radial distention of 5% and a shear stress of 10 dyn/cm² at a frequency of 60 cycles per min for up to 24 h [51]. Histological analysis revealed that the majority of MSCs responded to the mechanical stimuli by aligning within 20° to the direction of flow and by adopting a compact cell size, as is characteristic of ECs. However, gene expression and immunofluorescence analysis of both the stimulated and static groups reported that the cells did not express EC marker vWF factor on either the mRNA or protein level. Instead, the mechanically stimulated cells exhibited greater levels of smooth muscle-associated markers SMA and calponin in comparison to static samples. These results implicate the potential of MSCs as a cell source for the generation of SMCs, although the morphology and orientation appeared to resemble that of ECs.

In addition to stimulating differentiation, multimodalities of mechanical strain and shear could also affect matrix deposition by MSCs. When ovine bone marrow-derived CD45- and CD14-negative MSCs were cultured on poly(glycolic acid) (PGA) and poly(l-lactic acid) (PLLA) scaffolds in the presence of both cyclic stretch (60 cycles per min) and shear stress (~1 dyne/cm²) for 3 weeks, a 75% higher collagen content was observed compared with cells with stretch, shear or static control groups [52]. These results suggested that that combined stretch and shear could stimulate MSCs to remodel the matrix microenvironment, which has applications for accelerating tissue formation. These results together demonstrate the effect of shear stress and strain in modulating stem cell phenotype and behavior.

Role of matrix factors

Matrix materials are often used in the development of engineered vascular tissue constructs, but our understanding of the role of cell–matrix interactions is not well understood. To engineer matrix environments for cellular ingrowth, a number of naturally derived (i.e., collagen, laminin, fibronectin, Matrigel™, hyaluronic acid and fibrin) and synthetic polymers (i.e., PLLA and PGA) have been used [53–62]. Besides serving as a temporary scaffold for cell attachment, migration and survival, they may also modulate cell behavior and function based on the matrix rigidity and physical structure [63,64].

■ Role of matrix rigidity

Physiological matrix rigidity, a bulk property of the material that is characterized by the Young's modulus, varies within three orders of magnitude from soft tissues of the brain to hard tissues of the bone [65]. In particular, matrix rigidity plays an important role in cardiovascular applications, as cardiovascular cells respond to changes in matrix stiffness due to injury or disease such as MI or atherosclerosis. To utilize MSCs as a potential cell source for cardiovascular treatments, it is therefore important to understand the effects of matrix rigidity on MSC function and behavior as well.

The ability to sense matrix rigidity relies on mechanosensing structures in the cell such as integrins and focal adhesions. Integrins are transmembrane glycoproteins that serve as mediators of communication between the intracellular and extracellular environment from the inside-out or outside-in [66]. Structurally, they consist of an α - and β -subunit that assemble together in different combinations to form heterodimers. In human MSCs, α 1 through α 6 and β 1 through β 4 are reportedly expressed [67,68]. Binding of the extracellular domain of integrin mediates cell–cell and cell–extracellular matrix (ECM) adhesion, whereas binding of the cytoplasmic domain to intracellular ligands can activate signaling pathways or modulate cytoskeletal assembly. Related to integrins are focal adhesions, a complex of cytoskeletal proteins and enzymes that can bind the cytoplasmic domain of integrins or to actin [69]. Together, integrins and focal adhesions mediate the transduction of external cues that regulate cell morphology, migration, proliferation and differentiation.

To study the role of matrix rigidity, Pelham and Wang developed a method to vary the stiffness by changing the concentration of acrylamide and *bis*-acrylamide crosslinker used to form the matrix, while maintaining the same chemical composition [70]. This model enabled well-controlled studies of the effects of rigidity independent of chemical composition. Their studies, which assessed the role of substrate rigidity on fibroblast migration, led to striking observations that rigidity alone could modulate cell morphology and spreading area. In particular, they found that cells on softer substrates had lower spreading areas and faster migration rates, in comparison to cells on more rigid substrates [71]. Besides controlling cell spreading, matrix rigidity can also modulate the directionality of migration, as cells tend to migrate from soft (14 kPa) to hard (30 kPa) substrates [72].

Besides modifying the behavior of differentiated cells, rigidity also has profound effects on the fate of MSCs. Engler *et al.* demonstrated that human bone marrow-derived MSCs differentiated on polyacrylamide substrates towards osteogenic, muscular and neuronal lineages according to the physiological rigidity of respective tissues [73]. In particular, soft matrices that mimic brain tissue stimulated neurogenesis, whereas rigid matrices that resemble the stiffness of bone induced osteogenesis. These findings were supported by transcriptional profiling data showing upregulation of neurogenic markers on soft gels (0.1–1 kPa), myogenic markers on intermediate gels (11 kPa) and osteogenic markers on stiff gels (34 kPa). Lineage specification by matrix rigidity appeared to be dependent on non-muscle myosin II, as demonstrated by the loss of lineage specificity when cells were exposed to blebbistatin, an

inhibitor of non-muscle myosin II. However, further research is necessary to understand the role of matrix rigidity in modulating MSC morphology, migration and vascular lineage specification.

■ Role of matrix patterning

In addition to matrix rigidity, matrix patterning can also direct cell organization and differentiation [63]. Therefore, to mimic physiologically ordered vascular tissues, micropatterning and nanopatterning tools can enable well-controlled studies of restricted cell alignment. Soft lithography is a convenient method to generate micropatterns for biological applications. The desired pattern can be generated by photolithography onto a silicon wafer, which can be transferred to an elastomeric material such as PDMS [74]. Cells grown directly on the PDMS stamp would be subjected to the topographical features. Alternatively, the patterns on the PDMS stamp can be transferred onto other substrates by microfluidic patterning or microcontact printing.

Using microfluidic patterning of collagen I in parallel microchannels, we have previously shown that vascular SMCs can align according to the direction of the channels [75]. Furthermore, the proliferation rate of SMCs decreased on substrates with channels that were 20 and 30 μm wide, compared with nonpatterned substrates. In addition to SMCs, skeletal myoblasts could also align according to topographically patterned PDMS and form globally aligned myotubes in the direction of the microchannels [76]. Myotube length was significantly longer on micropatterned substrates than that on nonpatterned substrates, suggesting a potential role of micropatterning as a tool for generating physiologically aligned cellular constructs for tissue-engineering applications.

While the effects of micropatterning on vascular and muscle cells have been studied, the effect of micropatterning on MSCs remains to be elucidated. McBeath *et al.* employed micropatterned islands of fibronectin to probe the role of cell shape in mediating human bone marrow-derived MSCs towards adipogenic and osteogenic differentiation [77]. Notably, adipogenesis was associated with small ($1024 \mu\text{m}^2$) islands in which the cells remained rounded in morphology, whereas large ($10,000 \mu\text{m}^2$) islands preferentially induced osteogenic differentiation and an adherent morphology. It was further demonstrated that actin assembly and Rho GTPase mediate the differentiation of MSCs towards adipogenic and osteogenic lineages.

In addition to microscale features, nanoscale features can also restrict cellular alignment, behavior and differentiation. Nanofibrous matrices can be prepared by electrospinning, a process that generates nanoscale fibers formed by high-voltage electrostatic field [78]. In the electrospinning process, a thin stream of electrically charged polymer solution travels to a collector plate as it evaporates, forming fibrous polymer strands [78]. When a rotating drum is used as the collector plate, parallel-aligned fibrous networks can be generated [79]. Alternatively, randomly oriented fibers can be stretched to generate aligned fibers.

We have utilized electrospinning technology to generate parallel-aligned PLLA substrates to create aligned skeletal myoblasts, neurite outgrowth and MSCs [76,80,81]. In particular, we have shown that CD105- and CD166-expressing human bone marrow-derived MSCs cultured on aligned PLLA nanofibers with diameters similar to that of collagen fibrils (500–1000nm) remain viable and align in the direction of the nanofibers in vascular tissue-engineering applications [81]. Moreover, the cellular organization of MSCs was similar to that of SMCs on native arteries, suggesting that the nanofibrous substrates could mimic the structure native collagen matrix upon which SMCs are grown, which can be used to construct tissue-engineered vascular grafts.

Therapeutic potential of MSCs for vascular regeneration

■ Vascular grafts

In 2005, almost 500,000 coronary artery bypass procedures were performed [1]. Despite the improvement in overall quality of life by bypass procedures, there is still a growing need to engineer off-the-shelf vascular graft replacements to eliminate the need for donor vessels, which have a 35% 10-year failure rate. Synthetic acellular vascular grafts, which can be made of various biomaterials such as poly(ethylene terephthalate) and polyurethane [82,83], have been restricted to sizes larger than 5 mm in diameter owing to the heightened risk of thrombosis and occlusion associated with smaller graft diameter sizes. As a result, engineering living blood vessels is a promising alternative.

Blood vessels consist of three cell layers, namely an endothelial lining in the innermost layer, vascular SMCs in the medial layer and a fibroblast adventitial outermost layer. Models of living vascular grafts have been developed to contain cells alone, matrix alone, or the combination of cells, soluble factors and matrix [81,84–88], and the grafts may consist of multiple layers or single layer of cells and/or matrix (Figure 2). For example, Weinberg *et al.* showed that a piece of blood vessel can be constructed with ECs as inner lining and SMCs in collagen gel, supported by a Dacron mesh [84]. L'Heureux *et al.* demonstrated the feasibility of generating trilaminar vascular grafts without the use of a scaffold. The authors grew sheets of SMCs and wrapped them around a tubular support to produce the medial layer. Then, in a similar manner, they wrapped a sheet of fibroblasts around the SMC layer to generate the adventitial layer, before ECs were finally seeded in the lumen [86]. The same approach has recently been used to construct a bilayered vascular grafts containing ECs and fibroblasts [89]. Niklason *et al.* demonstrated the feasibility of engineering vessels by culturing ECs and SMCs in a tubular PGA porous scaffold in a bioreactor [87]. The engineered vessels were mechanically robust and could respond to pharmacological agents like native vessels. When implanted *in vivo*, the engineered arteries remained patent for up to 24 days.

Whilst the use of ECs and SMCs for generating vascular conduits would require patient-specific cell isolation, MSCs are a favorable alternative cell source because of their ability to be delivered allogeneically as an off-the-shelf therapy. Studies to date provide encouraging results that suggest that MSC-seeded grafts may be a feasible therapeutic option as a vascular conduit (Table 4). In one study, green fluorescence protein (GFP)-labeled rat bone marrow-derived MSCs that express CD90 and CD73 were cultured on polyurethane vascular prosthesis and assessed for their ability to differentiate into vascular lineages *in vivo* [90]. The cells were found to be organized in a multicellular structure that mimics that of native arteries 2 weeks after implantation in mice. The cells co-expressed GFP and SMC markers, suggesting that the *in vivo* setting induced smooth muscle protein maturation. MSC-seeded grafts were also more highly endothelialized than acellular grafts to prevent thrombosis. However, the electrophysiological profile of MSC-differentiated SMCs appeared to be different from that of mature SMCs. These results suggested that only partial differentiation of MSCs into SMCs were achieved by *in vivo* implantation.

We have recently shown that CD105- and CD166-expressing human bone marrow-derived MSCs that were cultured on aligned electrospun nanofibrous PLLA conduits can promote efficient *in vivo* infiltration of vascular cells and ECM remodeling [81]. *In vivo* transplantation of the MSC-seeded or acellular conduits as interpositional grafts in rat common carotid arteries showed that the cell-seeded grafts enabled long-term patency with well-formed layers of endothelial and smooth muscle layers. Both the cell-seeded and acellular grafts were shown to have extensive collagen deposition, but only cell-seeded grafts developed an elastic lamina adjacent to the lumen, implicating that the ECM and cellular components of MSC-seeded grafts closely resembled those in native vessels. Interestingly, only few MSCs could be found in the

grafts after explantation at 7 days after transplantation, suggesting that the long-term retention or differentiation of MSCs may not be necessary to confer the therapeutic benefits of anti-thrombogenesis and vascular remodeling.

The patency of MSC-seeded grafts may be related to the anti-thrombogenic properties conferred by MSCs [81]. Our *in vitro* studies demonstrated that platelet adhesion and aggregation was notably higher on SMCs than on MSCs or ECs, indicating that MSCs are comparable to ECs in their ability to confer antiplatelet adhesion properties. The mechanism of resisting platelet aggregation appears to be related to MSC cell surface heparin sulfate proteoglycans. The disruption of heparin sulfate proteoglycans by heparinase treatment led to increased platelet adhesion. This study demonstrated an important role of heparin sulfate proteoglycans on MSCs in resisting platelet adhesion.

Recent studies have utilized genetic modification of MSCs to better mimic the function of vascular cells. Kanki-Horimoto *et al.* transduced rat bone marrow-derived MSCs with an adenovirus harboring endothelial nitric oxide synthase (NOS), which is normally released by the endothelium to prevent thrombogenesis, to better mimic ECs [91]. While culturing the modified or naive MSCs onto small-caliber expanded polytetrafluoroethylene vascular prostheses, they monitored NOS activity based on the conversion of radiolabeled arginine to citrulline and found that the citrulline levels were increased in modified MSCs, in comparison to the naive cells. Furthermore, NOS activity could be blocked by the NOS inhibitor, *N*(G)-nitro-L-arginine methyl ester. This study highlights a promising technology for developing grafts with improved patency rates.

Tissue-engineered vascular grafts have also been shown to be mechanically durable. One study assessed the mechanical properties and therapeutic potential of a tubular scaffold consisting of a compact polyurethane layer sandwiched by porous PLGA on either side [92]. The burst strength of vascular grafts ranged from 160 to 183 kPa, which was similar to the burst pressure in veins (~223 kPa) [86]. In addition, the compliance was 0.57–53.80%/100mmHg, which was higher than that of conventional Dacron grafts. This tubular scaffold was next seeded with canine bone marrow stromal cells purified by Ficoll gradient centrifugation and implanted for up to 24 weeks in the canine abdominal aorta. Histological analysis of the vessels after explantation demonstrated full patency and differentiation towards EC and SMC lineages. These results suggest that this trilaminar scaffold favors mechanical strength and patency. Together, these studies demonstrate the therapeutic potential of MSCs and polymer scaffolds for developing vascular conduits that confer mechanical strength, patency and vascular differentiation potential.

Besides MSCs, endothelial progenitor cells (EPCs) can be used to cover the luminal surface of vascular grafts and prevent thrombus formation [93–98]. However, this approach may only be used autologously owing to the potential immune responses and the limited expansion capability of human ECs and EPCs. Alternatively, if appropriate adhesion or receptor ligands are available on the vascular graft, EPCs in the circulating blood can attach and cover the luminal surface [99,100]. Modification of vascular grafts to recruit EPCs or MSCs from circulating blood will be an attractive approach to facilitate the endothelialization of the vascular graft.

■ MSCs for treatment of MI

Besides generating vascular conduits, MSCs have been utilized for treatment of MI, which results when blood supply to the heart is blocked, leading to cardiomyocyte cell death. The pathological remodeling process that results includes scar tissue formation and thinning of the free wall, which contribute to the impairment of cardiac performance [101–103]. To repair MI tissue, angiogenesis is initiated to restore blood supply to the tissue. The process of

angiogenesis begins when supporting cells known as pericytes are first removed from the branching vessel. Next, the endothelial basal lamina and ECM degrade, and are replaced by a new matrix that attracts EC migration and proliferation. The ECs then form a tube-like structure that recruits pericytes and SMCs to the abluminal surface, forming a new vascular branch [104].

Angiogenesis is a highly organized process that is mediated by soluble chemical cues. For example, VEGF induces vascular hyperpermeability, endothelial migration and proliferation. FGF mediates endothelial migration, proliferation and tube formation, and TGF- β plays an important role in matrix remodeling and vessel restabilization [104]. Since these soluble factors play an important role in vascular remodeling and angiogenesis, they have become widely studied as potential agents to modify MSC behavior and function for vascular repair.

Various cell types have been used to repair MI tissue, including fetal cardiomyocytes [105], skeletal myoblasts [106], embryonic stem cells [107] and MSCs [108]. The modes of delivery into ischemic tissue include systemic or local routes including local injection, systemic injection or the placement of a local patch. Systemic delivery into the circulation can be advantageous because of minimal invasiveness, but the efficiency of directed homing may be limited. On the other hand, local delivery to the ischemic tissue is preferable to ensure cell transplantation to desired tissue locations, although accessing the myocardium may be surgically invasive. Cells can also be delivered concomitantly with polymers, which provide structural support and mediate cell survival and proliferation. Furthermore, the polymers can be adapted for delivering other factors, including cytokines, plasmids, viruses and drugs [109].

For the repair of MI, numerous studies report a therapeutic effect of delivering MSCs to improve cardiac repair by stimulating neovascularization and vascular differentiation (Table 4 & Figure 3). For example, the delivery of density-gradient purified bone marrow-derived canine MSCs for treatment of chronic myocardial ischemia led to significant improvement in ejection fraction and vascular density in the cell-treated group, and MSCs were also found to express EC and SMC markers [110]. Schuleri *et al.* delivered density-gradient purified bone marrow-derived porcine MSCs in allogenic models of MI and monitored myocardial blood flow by first-pass perfusion MRI over 8 weeks [111]. Their results demonstrated a significant improvement in myocardial blood flow over the first week in MSC-treated animals, compared with placebo-treated animals and untreated animals, but this improvement was not sustained at 4 and 8 weeks. On the other hand, global cardiac function was improved at week 8. This finding suggests that early tissue perfusion appears to precede improvement in cardiac function. To address the therapeutic potential of MSCs derived from patients with ischemic heart disease, CD105- and CD106-expressing bone marrow derived-MSCs from the diseased patients were delivered to immunocompromised mouse models of MI [112]. MRI assessment and immunohistochemical analysis showed that, after 14 days, the ejection fraction was significantly higher in the group receiving cell treatment. In addition, the MSC-treated group had increased vascularity and reduced thinning of the infarct scar, along with the generation of MSC-derived cells that expressed endothelial marker vWF. The therapeutic benefits of improved cardiac function and cardiomyocyte survival after MSC treatment has been confirmed by others as well [113–115].

Some reports raise concerns over the incidence of MSC differentiation into cardiovascular cell types as artifacts of cell fusion [116,117]. One study utilizing Cre/lox recombination to detect cell fusion events of murine bone marrow-derived MSCs showed that the cells fused *in vitro* with neural progenitors [116]. In the same study, *in vivo* bone marrow transplantation demonstrated that the cells fused with hepatocytes, Purkinje neurons and cardiac muscle, but no evidence of differentiation could be detected. However, other evidence points to

differentiation as a true phenomenon. For example, Kajstura *et al.* infused c-kit-expressing bone marrow-derived cells from male transgenic mice that expressed enhanced GFP (eGFP) into female recipient hearts [118]. Using both immunofluorescence detection of eGFP and fluorescence *in situ* hybridization for X and Y chromosomes, the authors detected newly formed cardiac and vascular cells that had one set of the X or Y chromosome as well as expression of eGFP. This study suggests that the cells differentiated rather than fused with host cells. Based on these studies, the relative contribution of cell fusion and differentiation is unclear.

Preservation of endogenous ECM and intercellular junctions also appears to be involved in promoting therapeutic benefits after MSC delivery. Miyahara *et al.* derived rat MSCs from adherent adipose tissue for transplantation as a patch onto the epicardial surface of infarcted hearts [119]. Their results showed that the sheets engrafted and grew in thickness, containing MSC-derived vascular cells, undifferentiated cells and few cardiomyocytes. The cell sheets also stimulated the formation of vascular structures originating from the host tissue, suggesting that MSCs could stimulate angiogenesis by acting through a paracrine mechanism. Another study comparing the therapeutic effect of rat MSCs derived from bone marrow by Percoll gradient centrifugation in either dissociated or sheet fragment form for repair of the infarcted heart reports significant enhancement of vascular density and graft/host cell connection for the cell sheet-treated animals [120]. The MSC sheet fragments were found to be aligned along the interstices of the injection sites, whereas very few cells were retained in the dissociated cell group.

The mechanism by which MSCs improve neovascularization after MI is unclear. Increasing reports provide evidence that MSCs act through a paracrine mechanism, in addition to differentiating into vascular lineages that directly contribute to vascular function. MSCs have been shown to secrete angiogenic factors such as VEGF and bFGF that may stimulate angiogenesis in ischemic tissues [121]. Furthermore, Tang *et al.* showed evidence that MSC-treated infarcts had higher levels of stromal cell-derived factor-1 α , a stem cell homing factor, as well as reduced levels of proapoptotic protein Bax, when compared with rats receiving media treatment [122]. Therefore, it is likely that MSCs are involved in inducing angiogenesis and promoting survival cues in ischemic tissues.

In addition to cells alone, MSCs delivered in conjunction with polymers have resulted in therapeutic improvement. In one recent study, a cardiac patch was formed by embedding human bone marrow-derived CD44-negative MSCs in collagen I matrix [123]. Transplantation of the construct as an epicardial patch for immunocompetent rats after MI led to the reduction of left ventricular interior diameter at systole, thickening of the anterior wall and improvement in cardiac function as assessed by fractional shortening. Interestingly, the cells were not detectable at 4 weeks after transplantation, suggesting that sustained cell retention was not necessary for cardiac improvement. A similar study of transplanting an epicardial patch consisting of multilayered rat bone marrow-derived MSCs in a decellularized pericardial scaffold also led to improved heart function and the formation of MSC-derived neovasculature [124]. We have also delivered bio-compatible matrices to the infarcted myocardium as acellular matrices or in conjunction with cells [64,125]. We demonstrated that local injections of collagen I, Matrigel and fibrin into the infarcted rat myocardium induced significantly higher capillary density, in comparison to saline control treatment, and could stimulate cell infiltration [64]. Whether or not the induction may be related to matrix-mediated activation of angiogenic factors or prosurvival cues needs to be further examined. Together, these studies illustrate the therapeutic potential of MSCs for increasing neovascularization and vascular differentiation, as well as improving cardiac function.

Another area of research interest is in the improvement of MSC viability after transplantation into the ischemic myocardium. Owing to the low survival rate of transplanted cells, Mangi *et al.* showed that the overexpression of Akt1 could improve MSC survival *in vitro* and *in vivo* [126]. Using rat CD34-negative bone marrow-derived MSCs that were retrovirally transduced with Akt1 containing a GFP reporter construct, the authors found that *in vivo* delivery of these modified cells maintained both higher cell retention and higher viability at 24 h after cell delivery into the infarcted heart, in comparison to cells modified with GFP reporter construct alone. In addition, the cells improved cardiac function and were found to express cardiac phenotypes. Related approaches to enhance MSC survival in the ischemic heart by either hypoxic preconditioning or by genetic modification of bcl2 or angiogenin expression have also resulted in higher cell retention *in vivo* [127–129]. These studies highlight the utility of genetic modification or preconditioning to improve transplant viability.

Conclusion

In summary, stem cell therapy is an exciting field of research for regenerative medicine, particularly for the treatment of cardiovascular diseases. MSCs are a promising cell source because of their ease of isolation and expansion, anti-thrombogenic property, low immunogenicity and potential to differentiate into cardiovascular lineages. In order to utilize MSCs for engineering vascular conduits or as treatment for MI, it is important to understand the role of the microenvironment in modulating MSC behavior and function. In particular, soluble factors, mechanical stimulation and matrix factors all have been shown to stimulate MSC differentiation along cardiovascular lineages *in vitro*. On the other hand, MSCs can secrete soluble factors and actively participate the remodeling process. The knowledge gained from this two-way signaling process will provide rational basis for the construction of vascular grafts and the treatment of MI.

Future perspective

Despite the progress made in engineering vascular conduits and in developing stem cell-based therapies for the treatment of MI, the clinical potential of MSCs remains not fully tested. Understanding the role of chemical and physical cues in the regulation of MSC survival, differentiation and tissue formation and the effects of MSCs on the remodeling process will be important for regenerative medicine. For example, optimizing matrix rigidity, chemical composition and mechanical environment that stimulates or sustains cardiovascular cells in both 2D and 3D environments will be important. Furthermore, elucidating the mechanism of their therapeutic effect *in vivo* will be necessary, as MSCs may be involved in the release of angiogenic factors to diseased tissues as well as in the incorporation into vascular structures. To this end, noninvasive techniques to tracking transplanted cells, such as bioluminescence imaging, MRI and PET, may be beneficial tools. Although autologous cell therapy is preferred in many clinical applications, whether allogenic MSCs can be used to fabricate vascular grafts or other tissue constructs to make them available off-the-shelf needs further investigation. More preclinical trials are necessary to understand the therapeutic effect of allogenic MSC grafts *in vivo*. Such studies will bring us closer to realizing the therapeutic potential of MSCs for regenerative medicine and cardiovascular tissue engineering.

Executive summary

Characterization of mesenchymal stem cells

- Although mesenchymal stem cells (MSCs) occupy only 0.01% of the total population of nucleated cells in bone marrow, *in vitro* they have a high expansion ratio of nearly 1 million-fold, while maintaining multilineage differentiation capacity. There is also evidence to show that MSCs have low immunogenicity.

- Currently, the populations referred to as MSCs do not have characteristics or isolation methods that researchers agree upon.

Role of soluble chemical factors

- Soluble factors such as VEGF, basic FGF, TGF- β and PDGF play a role in inducing MSC migration, proliferation and differentiation into vascular cell types.

Role of mechanical factors

- Shear stress and mechanical strain are mechanical factors that have been shown to stimulate MSC differentiation towards vascular lineages.
- Further research is necessary to elucidate the mechanism of action by which mechanical factors influence MSC fate.

Role of matrix factors

- Matrix rigidity modulates MSC differentiation towards osteogenic, muscular and neuronal lineages according to the physiological rigidity of respective tissues. However, the role of matrix rigidity on MSC differentiation towards cardiovascular lineages needs further investigation.
- Micropatterning and nanopatterning are useful tools for guiding cell organization and studying the role of restricted cell alignment on MSC differentiation.

Therapeutic potential of MSCs for vascular regeneration

- MSC-seeded vascular grafts have tremendous therapeutic potential because MSCs have anti-thrombogenic property, can recruit vascular cells through paracrine signaling and can differentiate into vascular lineages.
- For treatment of myocardial infarction, MSCs have been shown to improve cardiac function, differentiate into vascular lineages and stimulate neovasculature formation. The mechanism of action appears to be related to MSC differentiation and the release of angiogenic growth factors.

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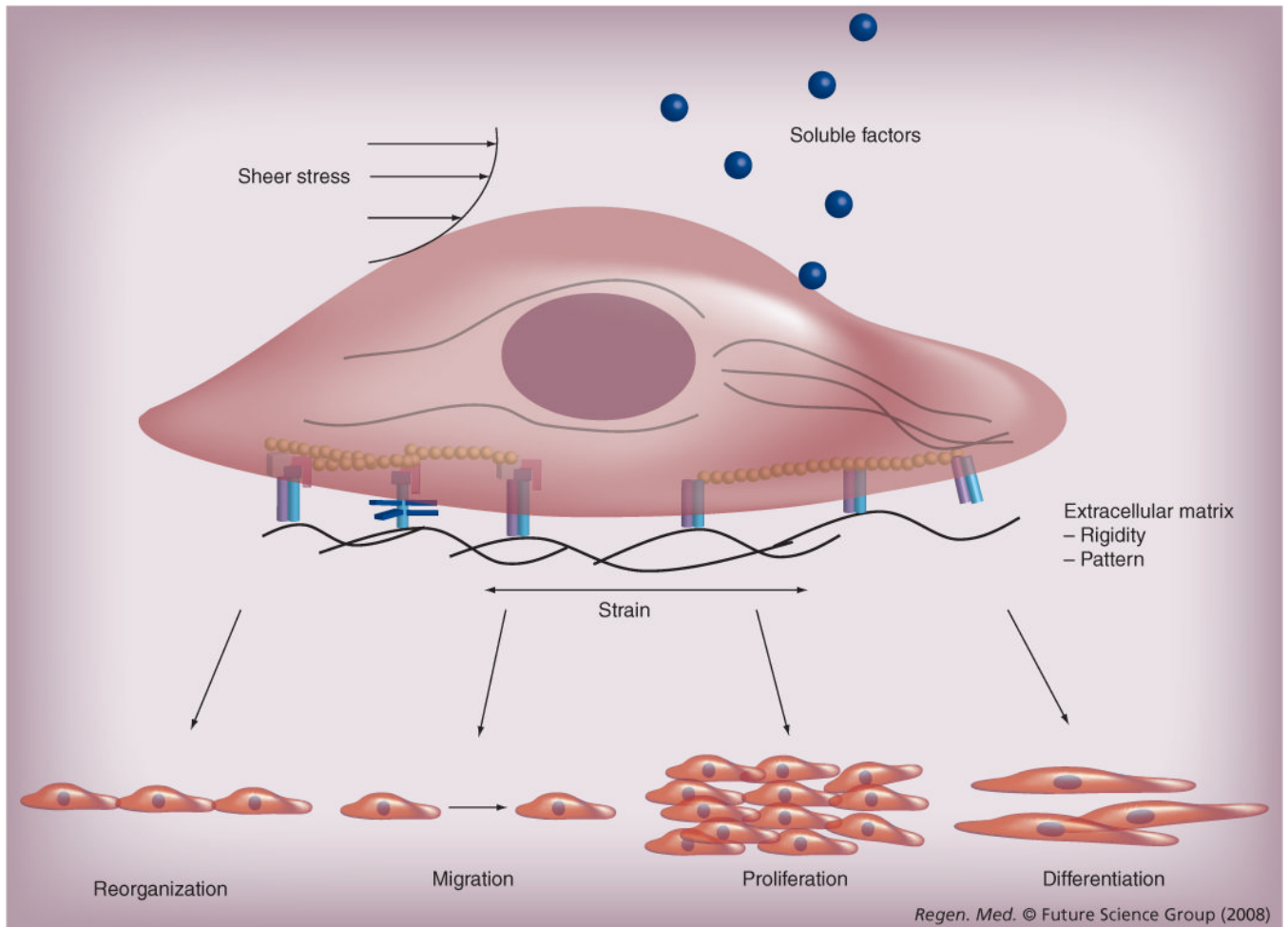


Figure 1. Multifaceted microenvironmental factors can affect mesenchymal stem cell phenotype and behavior.

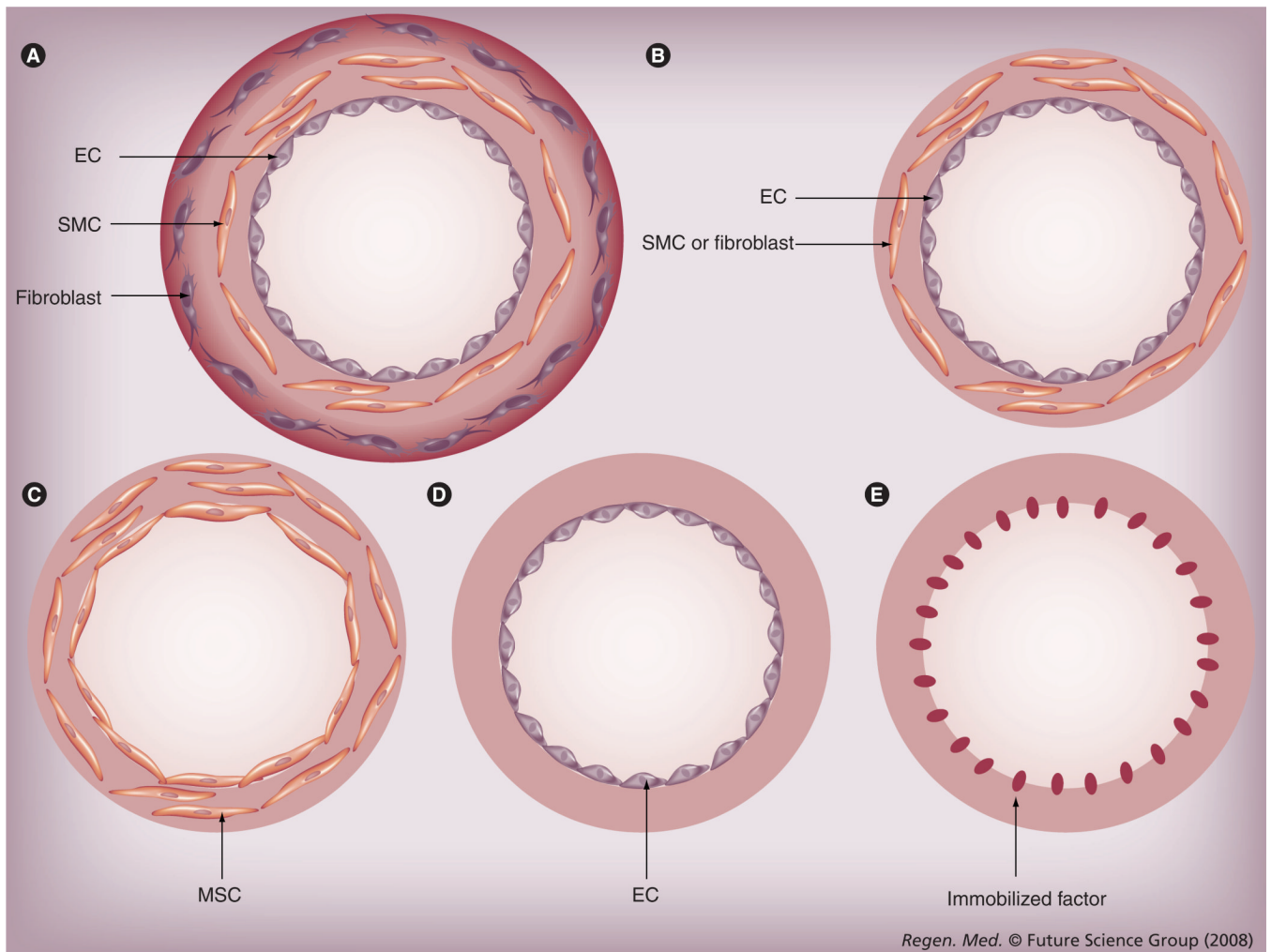


Figure 2. Models of vascular graft construction

(A) Three-layer model using ECs, SMCs and fibroblasts. (B) Two-layer model using ECs and SMCs/fibroblasts. (C) One-layer model using MSCs. (D) One-layer model using ECs. (E) Acellular model with immobilized factors.

EC: Endothelial cell; EPC: Endothelial progenitor cell; MSC: Mesenchymal stem cell; SMC: Smooth muscle cell.

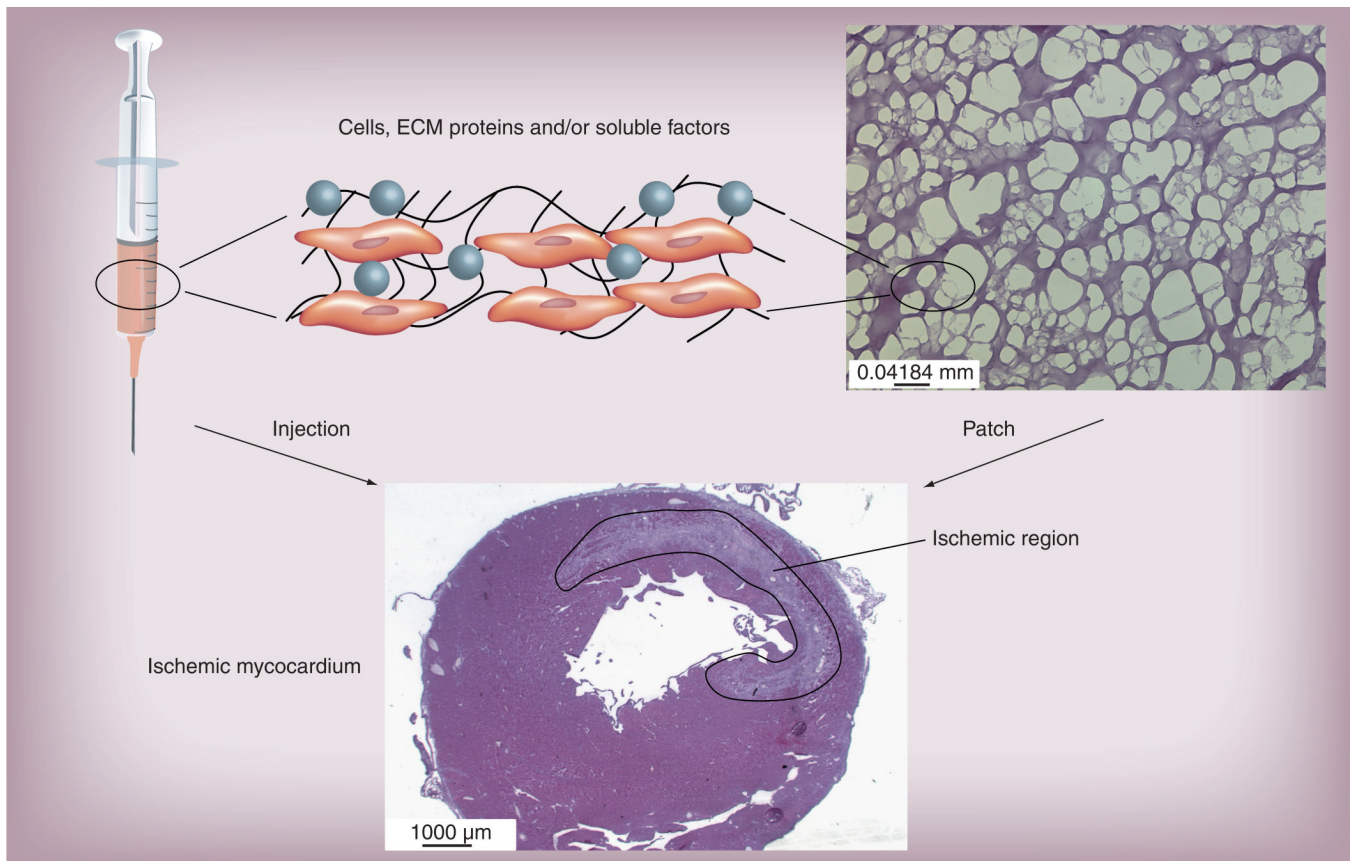


Figure 3. Models of cardiovascular repair

For treatment of the ischemic myocardium, cells and other factors can be injected directly to the center or border zone of the infarct. Alternatively, the cells can be transplanted as an epicardial patch using porous matrices such as fibrin. The solid line indicates the borders of the infarcted region.

ECM: Extracellular matrix.

Table 1

The effect of chemical factors on mesenchymal stem cell migration, proliferation and differentiation.

Chemical factor	Effect	Ref.
VEGF	Increases migration Increases proliferation Induces EC differentiation	[28–31]
PDGF	Increases migration Increases proliferation	[28,130]
bFGF	Increases migration Induces EC differentiation Induces SMC differentiation	[31,32]
TGF- β	Induces SMC differentiation	[33–36]

bFGF: Basic FGF; EC: Endothelial cell; SMC: Smooth muscle cell.

Table 2
Angiogenic factors produced by mesenchymal stem cells.

Factor	Induction with hypoxia ($\leq 1\% O_2$)	Ref.
Angiopoietin-1	-	[131]
bFGF	+	[131,132]
HGF	+	[132]
IL-1	+	[131]
IL-6	+	[131]
IGF-1	-	[132]
MCP-1	-	[131]
M-CSF	-	[131]
PIGF	+	[131]
PDGF	-	[131]
TGF- β	+	[131]
VEGF-A	+	[131,132]
VEGF-B	-	[131]

+: Induced in the presence of hypoxia; -:Not induced in the presence of hypoxia.

bFGF: Basic FGF; MCP: Monocyte chemoattractant protein; M-CSF: Macrophage-specific colony stimulating factor.

Table 3

The effect of mechanical factors on mesenchymal stem cells for vascular differentiation.

Mechanical stimulation	Effect	Ref.
Shear stress	Promotes EC differentiation	[46]
	Promotes SMC differentiation	[45]
Strain	Promotes SMC differentiation	[48,50]
	Inhibits SMC differentiation	[49]
Shear stress and cyclic strain	Promotes collagen deposition	[52]
	Promotes SMC differentiation	[51]

EC: Endothelial cell; SMC: Smooth muscle cell.

Table 4

The therapeutic potential of mesenchymal stem cells in *in vivo* for repair of myocardial infarction and vascular tissue engineering.

Application	Area of Improvement	Ref.
Vascular graft	Vascular differentiation Anti-thrombogenesis	[90,92] [81,91,133]
Myocardial infarction	Neovascularization Cardiac function Vascular differentiation Survival of transplanted cells	[110–112,119,120,122,124,127,128] [110–112,120,122,123] [110,112,119,120,124] [126–129]