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# Mesenchymal stem cells and vascular regeneration

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**Abstract**

In recent years, MSCs have emerged as a promising therapeutic cell type in regenerative medicine. They hold great promise for treating cardiovascular diseases, such as myocardial infarction and limb ischemia. MSCs may be utilized in both cell-based therapy and vascular graft engineering to restore vascular function, thereby providing therapeutic benefits to patients. The efficacy of MSCs lies in their multipotent differentiation ability toward vascular smooth muscle cells, endothelial cells and other cell types, as well as their capacity to secrete various trophic factors, which are potent in promoting angiogenesis, inhibiting apoptosis and modulating immunoreaction. Increasing our understanding of the mechanisms of MSC involvement in vascular regeneration will be beneficial in boosting present therapeutic approaches and developing novel ones to treat cardiovascular diseases. In this review, we aim to summarize current progress in characterizing the *in vivo* identity of MSCs, to discuss mechanisms involved in cell-based therapy utilizing MSCs, and to explore current and future strategies for vascular regeneration.

**KEYWORDS**

angiogenesis, mesenchymal stem cells, stem cell-based therapy, vascular regeneration

## 1 | INTRODUCTION

Cardiovascular disease is one of the leading causes of death worldwide and is in many cases a result of vascular injury. Conventional small molecule therapeutics to restore blood flow have proved to be only partially effective and more efforts are being invested in exploring the potential of stem cell-based therapy and use of engineered vascular grafts. Compared to the use of embryonic stem cells or induced pluripotent stem cells, the use of adult stem cells has the advantages of requiring less ethical consideration and the cells being less tumorigenic. Among adult stem cells, MSCs stand out because of the ease of their isolation and their relatively high growth rates and

short culture times, when compared to endothelial or other resident progenitor cells.

MSCs are plastic adherent cells isolated from various tissues and display multiple differentiation potentials *in vitro*, including the ability to differentiate into smooth muscle cells and endothelial cells, and can secrete various trophic factors, enabling them to contribute to vascular regeneration. Compared to conventional therapeutics, MSC-based therapy may benefit from two aspects. Vascular lineage cells differentiated from MSCs are able to directly take part in forming new vessels for blood flow restoration and trophic factors secreted by MSCs may enhance existing *in vivo* vascular regeneration capacity. While the paracrine effects are more commonly seen, more evidence is needed to establish the direct contribution of MSC-derived SMCs and ECs *in vivo*.

In addition to the direct cell-based therapies, MSCs also have potential in the development of bioengineered vessels for grafting. The three key components of such vascular grafts are cells, a biodegradable scaffold and humoral or mechanical signals.<sup>1</sup> A layer of endothelial

Abbreviations used: CFU-Fs, colony-forming unit fibroblasts; EC, endothelial cell; GEFs, guanine exchange factors; GPCR, G-protein-coupled receptors; HOP, homeodomain-only protein; IGF, insulin-like growth factor; ISCT, International Society for Cellular Therapy; JNKs, Jun amino-terminal kinases; MAPKs, mitogen-activated protein kinase; MRTFs, myocardin-related transcription factors; MSC, mesenchymal stem cell; PDGFRs, platelet-derived growth factor receptors; SMC, smooth muscle cell; SRF, serum response factor; TCE, TGF  $\beta$  control element; VEGF, vascular endothelial growth factor.

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cells within the scaffold has been shown to improve thromboresistance and clinical outcomes of vessel graft procedures<sup>2-4</sup>, while seeding of smooth muscle cells on a biodegradable graft can enhance in vivo arterial wall regeneration.<sup>5-7</sup> However, the limited life span and extended culture time of mature endothelial and smooth muscle cells has restricted their generalized use in tissue engineering. MSCs, with multi-lineage differentiation potential, are a valuable cell source for vascular engineering which do not suffer from these limitations. MSCs derived from the bone marrow are the most thoroughly explored type of MSCs.<sup>8-10</sup> Small diameter vessels have been engineered from bone marrow MSCs and exhibit substantial similarity to native vessels both at the histological and molecular levels.<sup>11</sup>

In this review, we discuss the utilization of MSCs in vascular regeneration through stem cell-based therapy and vascular graft engineering, explore the mechanisms of MSC contribution in vascular disease and repair and highlight animal and preclinical studies which examine the clinical feasibility of MSC application.

## 2 | BASIC CONCEPTS OF MSCS

### 2.1 | MSCs are found in various tissue types

Bone marrow MSCs were first discovered in the 1990s by the Caplan group, who utilized a diffusion chamber. This chamber is formed from two Millipore filters enclosed by a small plastic ring. Cells injected into the chamber remain inside the plastic as they cannot pass through the filters to contact the external environment. When transplanted into an animal, body fluids (nutrients, salts and proteins) can pass freely through the filters but there is no direct contact between host cells and the cells inside the chamber. After transplantation of the diffusion chamber into the peritoneal cavity of a nude mouse, the chamber is quickly surrounded by host vasculature, allowing it to serve as an in vivo incubator.<sup>12-14</sup> In addition to the use of a diffusion chamber, Caplan and his group also took advantage of porous calcium phosphate ceramics loaded with bone marrow cells, which they implanted at heterotopic sites (eg subcutaneously). They observed that bone marrow cells in these ceramics predominantly formed bone while bone marrow cells in plastic diffusion chambers formed a mixture of bone, cartilage, and fibrous tissue.<sup>14-16</sup>

By manipulating the culture conditions,<sup>17</sup> it was found that bone marrow cells can be induced into either adipocytic or osteogenic lineage. This was the first evidence for the in vivo and in vitro differentiation potential of bone marrow MSCs. Many subsequent studies have been conducted using various differentiation conditions which confirm that these cells can differentiate into adipocytes, osteocytes, and chondrocytes. It is worth noting that Caplan was also the first to call these cells multi-potent “MSCs”.

Since the isolation of these bone marrow MSCs with trilineage differentiation potential, cells with similar characteristics have been isolated from multiple human tissue types including adipose tissue, synovial membrane, periodontal ligament, tendon, skin, cartilage, dental pulp, eye, gut, heart, kidney, liver, lung, muscle, pancreas, spleen, thymus, umbilical cord, placenta, aorta, vena cava, cord blood, and peripheral blood.<sup>18-31</sup>

These cells have shown clinical benefits in a number of areas such as cardiovascular disease (myocardial infarction and ischemia), pulmonary disease, and neurological diseases. MSCs are also widely used in translational research, such as orthopedic reconstruction, which has promising clinical potential. Some clinical trials have taken advantage of the benefits of the immune regulative function of MSCs and have shown promising and reproducible results in immune disorders such as multiple sclerosis, graft-versus-host disease and Crohn's disease.<sup>32,33</sup>

### 2.2 | Minimal criteria for identifying MSCs

Since 2000, there has been an increase in the number of studies focused on MSCs. However, in the absence of well-established standard characterization method, obvious discrepancies have been reported between different laboratories. This has led to difficulties in accurate interpretation of the results. To address this problem, ISCT published a position statement on the minimal criteria to define multipotent mesenchymal stromal cells cultured from human tissues.<sup>32</sup> In this position paper, MSCs are defined as being adherent to a plastic surface when cultured under standard conditions and to acquire a specific phenotype and display multipotential differentiation capacity in vitro. (The minimal criteria for human MSC classification is summarized in Table 1) Surface marker expression of negative indicators CD45, CD34, CD14 or CD11b, CD79 $\alpha$ , and CD19 is used to exclude contamination of cultures with pan-leukocytes, primitive hematopoietic progenitors, monocytes/macrophages, and B cells, respectively. Positive surface marker expression analysis merits further exploration because none of the markers currently used are MSC specific. More markers must also be identified to allow researchers to distinguish between the differentiated and undifferentiated states of MSCs.

Though the ISCT position is a start, these criteria do not exclusively identify MSCs.<sup>33</sup> Sabatini identified human bronchial fibroblasts exhibiting a MSC phenotype and multilineage differentiation potential in 2005. Furthermore, though these markers are used to identify MSCs in vitro, they have no use when attempting to identify MSCs in situ. MSCs in culture may be very different from those in vivo, as expression of some markers may be an artifact of the culture process.

Other markers used in MSC characterization include positive expression of Stro-1,<sup>34</sup> which can enrich CFU-Fs by approximately 100 fold in human MSCs, in combination with a glycoprotein A negative

**TABLE 1** The minimal criteria for characterizing human MSCs

Basic Characteristics	Description
Plastic Adherence	Plastic adherent in standard culture conditions
Phenotype: positive ( $\geq 95\%$ ) surface markers	CD73, CD90, CD105
Phenotype: negative ( $\leq 2\%$ ) surface markers	CD45, CD34, CD14 or CD11b, CD79 $\alpha$ or CD19, HLA-DR
In vitro differentiation capacity	Osteoblasts, adipocytes, chondrocytes (demonstrated by staining of in vitro cultures)

phenotype (to exclude erythroid progenitors). However, Stro-1 is only expressed in human tissues and there is no corresponding marker in other species. Recent findings also indicate that Stro-1 is expressed in the endothelium rather than in mesenchymal tissue *in vivo*, and that it can be induced by *in vitro* MSC culture conditions.<sup>35</sup> Additionally, it is well documented that differences exist across tissue origins and between species. This serves as evidence that further work should be done to identify unique markers of MSCs.

### 2.3 | *In vivo* identity and function of MSCs

MSCs are a heterogeneous population and this is reflected by the colonies they form *in vitro*, which have different growth rates and morphologies, ranging from fibroblast like spindle-shaped cells to large spread cells. Furthermore, if colonies are allowed to grow for a long period, heterogeneity within the colonies can also be observed, with some of the colonies alkaline phosphate positive, some of them negative, and some others positive in the center and negative in the periphery region.<sup>36</sup> This heterogeneity might be explained by the fact that tissues themselves are made up of numerous cells and therefore have diverse precursor types and are heterogeneous in nature and origin. However, once isolated and in *in vitro* culture, these cells seem to display a number of similarities concerning phenotype and multilineage differentiation potential.

To minimize the discrepancy in reported properties of MSCs and to increase the consistency of molecular studies and preclinical trials between different labs, characterization of *in situ*/native MSCs is a prerequisite for their study and could allow for improved purification of these cells.

Approaches to searching for the *in vivo* environment, localization, and identity of MSCs attempted so far include seeking surface markers expressed *in vitro*, infusion of labeled MSCs *in vivo* to track their homing and distribution, and isolation and characterization of cells from various tissues to determine whether they have the reported properties of MSCs.

As discussed earlier, Stro-1 is highly expressed in stromal cells which are clonogenic in *in vitro* culture.<sup>37</sup> Theoretically, identifying cells expressing high levels of Stro-1 in *ex vivo* sections could identify the location of native MSCs, providing evidence for micro-anatomical niches or aiding identification of the clonogenic cells.<sup>38</sup> A previous study of frozen sections stained with Stro-1, found that the vascular wall was the main location for these positive cells. However, a major obstacle is that most surface markers expressed by MSCs cultured *in vitro* do not uniquely identify these cells and are also expressed by other cell types. For example, CD105 is expressed by endothelial cells and CD44 is expressed by smooth muscle cells. A further complication is that the phenotype of MSCs changes once they are cultured *in vitro*, which suggests that even if we could characterize cells with some specific markers they may not necessarily have clonogenic potential *in vitro*. As a result, characterization of MSCs *in vivo* based on their phenotypic features is like shooting a moving target, in that the phenotype of MSCs constantly changes in response to their *in vitro* and *in vivo* microenvironment.<sup>38</sup>

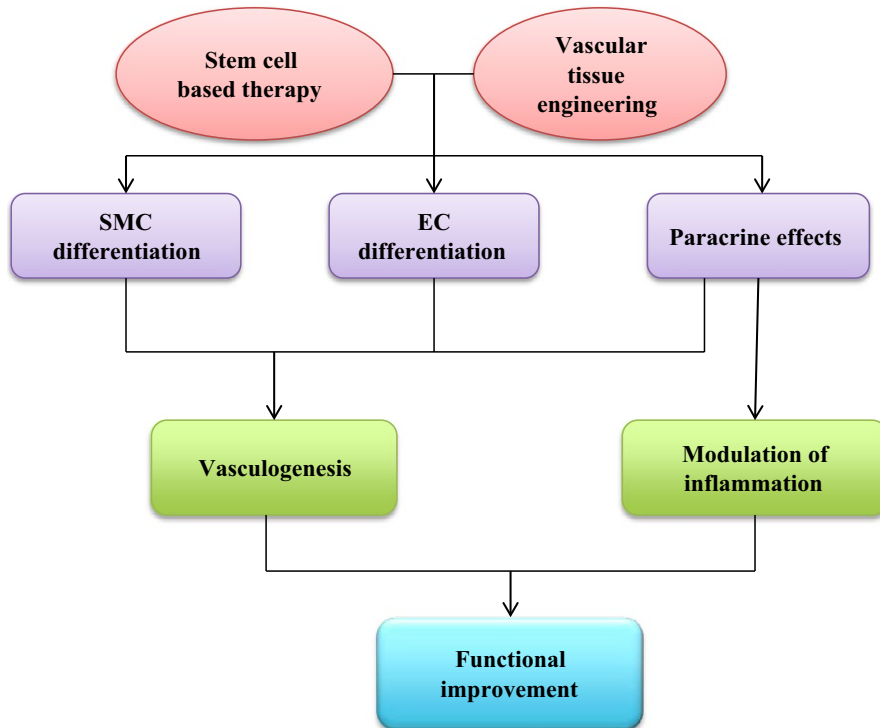
Another strategy is to infuse MSCs *in vivo* to track their engraftment and homing to specific tissues. After transducing murine bone marrow-derived MSCs with eGFP, labeled cells were injected systemically into minimally injured mice and tissue-specific differentiation of these cells was determined by RT-PCR and immunohistochemistry.<sup>39</sup> Contribution of donor-derived eGFP-MSCs to a number of different cell types including hepatocytes, lung epithelial cells, myofibroblasts, and renal tubular cells was observed. Although this approach can provide functional information about MSCs in tissue regeneration, utilizing it as a proof of the native localization of MSCs is inappropriate, as non-specific binding of MSCs in various tissues cannot be excluded.

Systematic isolation and evaluation of MSCs from different tissues represents an alternative approach.<sup>29</sup> It has been reported in a number of studies that MSCs with similar *in vitro* characterizations can be cultured from various tissues such as adipose tissue, umbilical cord, tendon, synovial membrane, and others. In one study, isolation of MSCs from tissues throughout the body (brain, kidney, liver, lung, spleen, bone marrow, vena cava, and aorta) provided an opportunity to consistently visualize the distribution of MSCs *in vivo*.<sup>29</sup> The similar though not identical phenotypes of MSCs originating from different tissues suggests that they may have similar origins but respond to differing microenvironmental influences. The proposed hypothesis that MSCs are tissue resident stem cells has led to further investigations into their perivascular origin,<sup>40</sup> which some studies had already suggested.<sup>28,38,41</sup> A series of recent studies suggest that human adipose tissue pericytes and MSCs share a similar gene expression profile.<sup>42,43</sup> Despite of the accumulating evidence indicating the perivascular origins of MSCs, disputes have arisen, some of which lie in the use of CD146 as a perivascular surface marker. CD146 is more a pericyte marker than a perivascular marker, and a population of CD146-negative perivascular cells which reside in the tunica adventitia also exhibit MSC characteristics. Other studies have shown the subtle functional differences between pericytes and MSCs, such as in angiogenic function<sup>44</sup>, engraftment potential,<sup>45</sup> and differentiation abilities<sup>42</sup>.

As none of the approaches used so far provide a definite and explicit answer to the question of the *in vivo* identity of MSCs, more studies should be done to track their development origin and elucidate their *in vivo* function.

## 3 | MECHANISMS OF MSC UTILIZATION FOR VASCULAR REGENERATION

Utilization of MSC as a therapeutic agent for vascular regeneration includes direct administration of MSCs either systematically or locally, to trigger and take part in vascular regeneration and indirectly grafting engineered vascular tissue seeded with MSCs or partially differentiated MSCs, to relieve ischemia and restore blood flow, thus preventing organ damage caused by hypoxia, inflammation, and plaque rupture.<sup>43</sup> In both cases, the therapeutic angiogenic potential of MSCs relies on their differentiation toward vascular lineages such as smooth muscle cells and endothelial cells as well as their paracrine effects. (Figure 1)



**FIGURE 1** Mechanisms of MSC utilization for vascular regeneration. MSCs contribute to vascular regeneration by either being administered systematically/locally in stem cell-based therapy or being utilized to construct vascular grafts. In both cases, their angiogenic potential relies on the differentiation capacity to smooth muscle and endothelial lineages as well as the paracrine effects. Functional improvement such as ischemia alleviation and blood flow restoration is achieved in the end through angiogenesis and modulation of inflammation

### 3.1 | Differentiation towards smooth muscle cells

Developmentally, depending upon their location, vascular smooth muscle cells originate from different embryonic stages, forming different vessels or different sections of one vessel with sharp boundaries.<sup>46,47</sup> In vitro protocols for SMC differentiation utilizing stem cells at different embryonic stages have been established,<sup>48</sup> which include treatment with all-trans retinoid acid (at-RA), TGF  $\beta$  and PDGF-BB, and culture on collagen IV, either alone or in combination. However, all these systems utilize in vitro culture of cells which is an intrinsic limitation. When interpreting experimental results, it must be considered that to what extent these in vitro systems recapitulate SMC differentiation in vivo remains unknown.

The adult stem cell pool includes bone marrow cells (hematopoietic stem cells and MSCs), which can be mobilized and released into the circulation, and tissue resident stem cells (vascular progenitor cells and stem cells from tissues all around the body). It is possible that cells from both of these populations may contribute to the SMC accumulation that is a major event in the development of atherosclerosis. For this reason, efforts are being invested in exploring the origin of SMCs at the neointima. In addition, it is now recognized that these SMCs may originate from transdifferentiation of other cell types such as endothelial cells<sup>49,50</sup> and fibroblasts<sup>51,52</sup> or from phenotypic switch of SMCs in the media.

Growing evidence is accumulating that suggests adult stem/progenitor cells differentiate into SMCs in a variety of cardiovascular disease settings, including atherosclerosis and intra vascular stent restenosis. A number of in vivo studies provide evidence of the origins of tissue resident stem cells that give rise to SMCs in the neointima.

In 2001, Han and colleagues<sup>53</sup> confirmed that bone marrow-derived cells could contribute to vascular healing, by providing an

alternative source of smooth muscle like cells when the media is severely damaged, however, they were unable to elucidate whether the cell type within the bone marrow taking effect was hematopoietic stem cells or MSCs. Later, Shimizu<sup>54</sup> confirmed the finding of Han by transplantation of bone marrow galactosidase expressing cells. Sata<sup>55</sup> et al. took the study one step further by injecting purified hematopoietic stem cells into the mouse circulation and observed that injected cells could participate in pathogenesis of atherosclerosis. Although they confirmed that hematopoietic stem cells had the potential to differentiate into SMCs in vivo and in vitro, they did not exclude the possibility that the MSCs within the bone marrow could be mobilized by vascular injury and take part in atherosclerosis.

Despite these studies, other conflicting data exist as to whether bone marrow is the source for neointima SMCs. Bone marrow transplantation of SM-LacZ beta-gal expressing cells into mice suggested that bone marrow progenitor cells did not serve as a SMC source.<sup>56</sup> Work from the same group also identified a type of Sca-1(+) cells within the adventitia of aortic root and confirmed that after in vitro expansion and culture when these Sca-1(+) positive cells were seeded onto the adventitia side at a site of vascular injury, the cells migrated into the media and intima and participated in atherosclerosis lesion formation and vascular repair.<sup>57</sup>

An explanation for these conflicting data is that SMA staining is more sensitive than SM-LacZ staining. Thus, the lack of a definitive marker of differentiated state SMCs means that the question of whether bone marrow-derived stem cells can contribute to SMC differentiation or not remains open.

As stated earlier, several processes may contribute to SMCs presence in atherosclerotic lesions, and it remains unclear to what extent stem cells could give rise to SMCs in pathogenesis. What is now widely

accepted is that the origin and residency of these stem cells requires further exploration.

## 3.2 | Mechanism of smooth muscle differentiation

### 3.2.1 | SRF–CArG-dependent regulation of SMC differentiation

Serum response factor is an MADS (MCM1, agamous, deficiens, serum response factor) box transcription factor, which binds to the highly conserved CArG cis-elements (CC(A/T)6GG) that are present within virtually all promoter or intronic sequences of SMC marker genes, including SMA, SM22, calponin, SMMHC, and desmin. SRF binds with CArG elements as a dimer and with the interaction of other co-factors such as myocardin, the MRTFs, and some Nkx and Gata family members. The resulting complex has the capability to regulate SMC marker expression. (Figure 2) SRF is a ubiquitously expressed protein, which also regulates cardiac and skeletal muscle gene expression, as well as expression of some early response and structural genes.<sup>58</sup> Although expressed widely in many cell types, the relatively high level of SRF within SMCs (and other muscle cell types) partly explains its specific regulation of muscle cell-specific genes.<sup>59,60</sup> The ability of SRF to bind to CArG regions of SMC genes proves to be the rate-limiting factor in SMC differentiation.<sup>61</sup>

Other than myocardin and the myocardin-related family, the homeodomain proteins (Hox, Nkx3.1, Prx-1, and Barx2b) also enhance SRF binding ability, while the HERP1/HEY2<sup>62</sup> (a target of the Notch signaling pathway), HOP, and YY1 possesses the ability to inhibit it.<sup>63–65</sup>

Post-translationally, modification of SRF by phosphorylation changes the binding affinity of SRF with transcription factors. Arginine vasopressin increases SM- $\alpha$  actin promoter activity by JNKs and p38 MAPKs-mediated phosphorylation of SRF at Ser103.<sup>66</sup> Phosphorylation at Ser162 by protein kinase C $\alpha$  and at Thr159 by protein kinase A decreases SM- $\alpha$  actin promoter activity.<sup>67,68</sup> Interestingly, CArG elements control the promoter activity of SRF,<sup>59</sup> suggesting a positive feedback loop in the control system.<sup>69</sup>

The SRF-CArG regulating pathway alone is not sufficient to trigger SMC-specific gene expression, other *cis* elements and trans-binding factors also play a very important role in the regulation of SMC differentiation, including the TCE, E box elements (CANNTG motifs), and a *cis* element referred to as a G/C repressor.<sup>70–72</sup> Mutation of TCE and E box elements, which are non-CArG elements, eliminated expression of SMC-specific genes in transgenic mice.<sup>70,73</sup>

In summary, SMC differentiation depends on the integration of multiple regulatory pathways, in which SRF-CArG *cis* elements regulation plays a critical but not exclusive role.

### 3.2.2 | The RhoA-regulated signaling pathway

RhoA has been proven to influence SMC differentiation in a variety of studies,<sup>74,75</sup> and RhoA kinases are important RhoA effectors. Various environmental cues can promote SMC differentiation through

the RhoA pathway including thrombin<sup>76</sup>, TGF $\beta$ <sup>77,78</sup>, S1P (sphingosine 1 phosphate)<sup>79</sup>, BMP<sup>80</sup>, and cell stretch<sup>81</sup>. These agonists activate transmembrane GPCRs and with subsequent facilitation by GEFs, which exchange GDP for GTP, RhoA is activated.<sup>82</sup> Details about the GPCR types and GEFs that respond to each agonist are reviewed elsewhere.<sup>83</sup>

Before RhoA activation, MRTFs bind to G-actin and remain in the cytoplasm. After RhoA-ROCK (RhoA kinases) pathway activation, however, G-actin in the cytoplasm forms F-actin and dissociates with MRTFs, releasing MRTFs and promoting their translocation from the cytoplasm to the nucleus. Increased numbers of MRTFs in the nucleus interact with SRF and enhance SMC-specific gene expression.<sup>84</sup> Direct evidence of this nuclear translocation pattern has been observed using enhanced GFP fusion proteins.<sup>85</sup> (The RhoA regulated signaling pathway is depicted in Figure 2)

### 3.2.3 | Epigenetic modulation and micro-RNA regulation

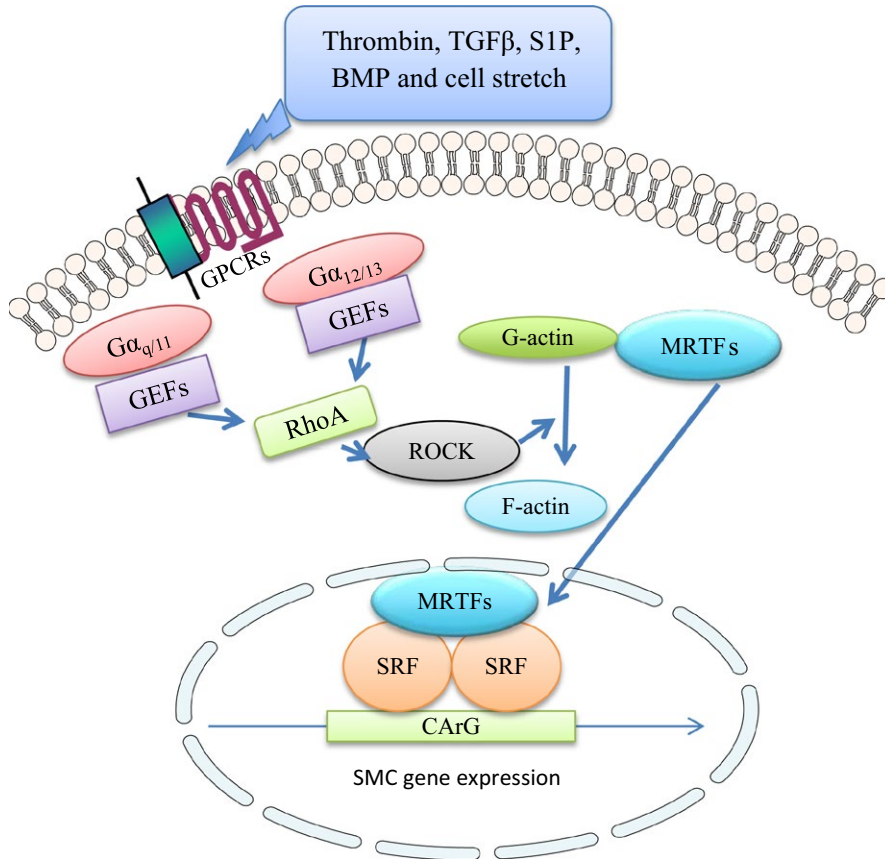
Genomic DNA is wrapped in a compact structure known as chromatin, which is composed of nucleosomes and connected by a linker chromatin. Histones (2 $\times$  H2A, 2 $\times$  H2B, 2 $\times$  H3, 2 $\times$  H4) and a 146 base pair long genomic DNA form the basic unit of nucleosome. The linker chromatin is formed by DNA of variable lengths and histone H1. The N terminal tail of histones is freely exposed to the environment and is prone to modifications including methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation.<sup>86</sup> Modification of histones or interaction of histones with other binding proteins can change protein conformational structure and the accessibility of DNA within the chromatin to transcription factors.

MicroRNAs are also necessary for the differentiation of SMCs, as demonstrated by the study of Dicer deletion experiments, which resulted in the failure of microRNA maturation.<sup>87,88</sup> Dicer deletion in vascular SMCs caused embryonic lethality at day 16 to 17, with extensive internal hemorrhage generated by reduction of SMC proliferation and impaired contractility, which could be partly rescued by overexpression of microRNA145.<sup>89,90</sup> Through the repression of antagonistic factor (Klf4, Klf5, Elk1, versican, and angiotensin converting enzyme) gene expression in the SMC differentiation process, microRNA-143/145 facilitates expression of SMC-specific genes.<sup>91–95</sup> MiR-133 is reported recently to be able to inhibit SMC phenotypic switch by repressing the transcription factor Sp1 and regulate the expression of smooth muscle genes.<sup>96</sup> In addition, miR-1<sup>97,98</sup>, miR-21<sup>99</sup>, and miR-10a<sup>100</sup> can also regulate smooth muscle differentiation.

### 3.2.4 | Differentiation potential of MSCs towards endothelial cells

MSCs from different sources have been shown to be capable of differentiation toward an endothelial lineage. Oswald and colleagues showed that human bone marrow-derived MSCs could differentiate toward angiogenic endothelial-like cells expressing KDR and vWF by





**FIGURE 2** The RhoA pathway regulates SMC differentiation in response to various environmental cues. Thrombin, TGFβ, S1P, and BMP bind to GPCRs signal through the RhoA-ROCK pathway, which results in polymerization of G-actin. MRTFs go on to dissociate with G-actin and are translocated to the nucleus, where they activate SMC gene expression by binding the SRF-CArG elements

simply culturing the cells in the presence of 2% fetal calf serum and 50 ng/mL VEGF for 7 d.<sup>101</sup> Using a canine chronic ischemia model, bone marrow-derived MSCs were shown to differentiate into an endothelial phenotype to facilitate revascularization and to improve heart function.<sup>102</sup>

Adipose tissue represents another important source of MSCs with EC differentiation ability. MSCs isolated from human adipose tissue can give rise to a CD34 and CD13-positive population which can spontaneously differentiate into endothelial cells in vitro and in vivo.<sup>103</sup> Miranville et al. defined a CD34-positive, CD31-negative cell population from adipose tissue that could differentiate toward ECs in the presence of VEGF and IGF.<sup>104</sup> More studies have further confirmed that MSCs isolated from adipose tissue have the capacity to differentiate into ECs.<sup>105</sup> However, Fischer et al. reported that although EC growth supplement and shear stress can induce certain endothelial characteristics in adipose-derived MSCs, the derived cells did not acquire the full endothelial profile.<sup>106</sup> Therefore, further modifications and evaluation are needed for clinical use of MSC-derived ECs as endothelial replacements. Care must also be taken to distinguish adipose tissue-derived MSCs with EC differentiation ability from mature ECs that already exist in adipose tissue.<sup>107</sup>

Umbilical cord-derived MSCs also demonstrate endothelial lineage commitment potential. MSCs isolated from human umbilical cord by explants avoided endothelial contamination and could differentiate into endothelial lineages in vitro on 2D or 3D gel scaffolds, as well as in vivo in an ischemic hindlimb mouse model to improve limb perfusion.<sup>108</sup>

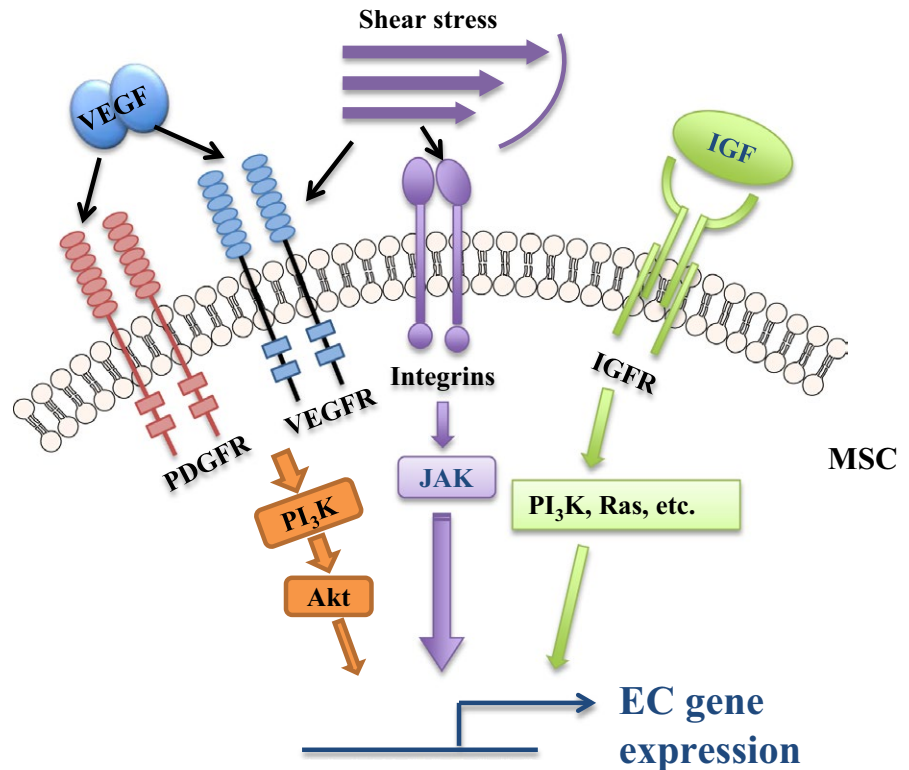
### 3.2.5 | Mechanisms involved in endothelial differentiation from MSCs

In addition to exploring the feasibility of using MSC as a cell population with endothelial differentiation potential, the underlying mechanisms regulating MSC to endothelial differentiation have also been studied. Understanding these important regulatory mechanisms can further help us to improve differentiation efficiency. (Mechanism of endothelial differentiation is depicted in Figure 3)

VEGF signaling is one of the most important signaling pathways that regulate vascular development and endothelial differentiation. Almost all protocols for MSC to endothelial differentiation include the supplement of VEGF in culture medium, to induce endothelial lineage commitment. However, the exact regulatory mechanisms of VEGF in MSC to endothelial differentiation are still under discussion. Although MSCs usually do not express VEGF receptors, it is reported that VEGF can generate downstream effects through PDGFRs.<sup>109</sup> The downregulation of PDGFRs in MSCs leads to strong inhibition of MSC to endothelial differentiation and MSC-mediated vasculogenesis.<sup>110</sup> In addition, there are also studies that suggest VEGF stimulation can activate VEGFR1 expression, which further induces endothelial differentiation.<sup>111,112</sup> A study showed that VEGF induced human and rat bone marrow-derived MSC differentiation to ECs through the Rho/ROCK signaling-mediated nuclear translocation of MRTF-A.<sup>112</sup>

Mechanical stimulation also plays important roles in regulating the differentiation directions of MSCs. Fluid shear stress can induce

**FIGURE 3** Mechanisms involved in regulating MSC to endothelial differentiation. Multiple chemical and mechanical stimuli regulate the differentiation of MSCs toward endothelial lineages. VEGF activates PDGF receptors and VEGF receptors to transmit the downstream signals which enhance EC gene expression. IGF can bind to IGF receptors to activate several endothelial-related cellular signaling pathways. Shear stress can trigger different sensors at the cell surface to initiate signaling which leads to endothelial differentiation



endothelial differentiation of multiple kinds of stem cells.<sup>113</sup> Bai et al. emphasized that a combination of VEGF treatment and shear stress stimulation can effectively induce endothelial differentiation of bone marrow-derived MSCs.<sup>111</sup> Another study showed a similar effect, in that a combination of shear stress and VEGF can enhance endothelial differentiation of human adipose tissue-derived MSCs.<sup>114</sup> By using a tissue-engineered vascular scaffold seeded with bone marrow-derived MSCs, Dong et al. showed that shear stress can induce an endothelial phenotype in MSCs.<sup>115</sup> 3D tubular collagen scaffolds also induce the differentiation of MSCs toward endothelial lineage suggesting structural environment may influence differentiation.

### 3.2.6 | Paracrine effects of MSCs

In studies exploring the mechanism of MSC therapy in ameliorating myocardial infarction, the differentiation potential of MSCs toward cardiomyocytes was observed, in spite of their effect in promoting neovascularization. However, the possible capacity of adult stem cells, including MSCs, to differentiate toward cardiomyocytes is a controversial topic and requires further investigation.<sup>116-119</sup> A paracrine mechanism of action was later proposed and further evidence showed that trophic factors released by MSCs could contribute to the regeneration of the ischemic heart.<sup>120-122</sup> It was also demonstrated that bone marrow-derived MSCs overexpressing Akt could improve cardiac function as early as 72 h after MSC administration, which could not be attributed to the cardiomyocyte differentiation ability of MSCs.<sup>121</sup> Moreover, conditioned medium from hypoxic Akt-MSCs could significantly attenuate ischemic damage to the heart. Consistent with the paracrine activity hypothesis, various trophic factors including VEGF,

bFGF, HGF, and IGF-1 have been shown to be upregulated in hypoxic Akt-MSCs compared to controls.<sup>122</sup>

Trophic factors secreted by MSCs include angiogenic factors, anti-apoptotic factors and immunomodulation factors.<sup>123</sup> MSCs express angiogenic factors such as VEGF and bFGF, as well as MCP-1 and SDF-1 which are essential for vascular network remodeling.<sup>120</sup> Optimal therapeutic angiogenesis is achieved with the participation of VEGF<sup>124</sup>, bFGF<sup>125</sup> and angiopoietin 1<sup>125</sup> secreted by MSCs. B cell lymphoma 2 (Bcl-2), a classical inhibitor of apoptosis, can be synthesized and secreted by MSCs and affect nearby cells<sup>126</sup> as well as themselves<sup>127</sup>. Other anti-apoptotic factors secreted include survivin and Akt.<sup>126</sup> Furthermore, MSCs can secrete human leukocyte antigen class I molecule G5 (HLA-G5) and display immunosuppressive properties.<sup>128</sup>

## 4 | ANIMAL STUDIES AND CLINICAL TRIALS USING MSCS FOR VASCULAR REGENERATION

Vascular regeneration therapies with small molecules are proven to be inefficient thus far. Therefore, efforts have shifted to alternative stem cell therapies such as treatment with embryonic stem cells, induced pluripotent stem cells, and adult stem cells.<sup>43</sup> Among adult stem cells, MSCs have recently emerged as an important candidate for vascular regenerative studies.<sup>129</sup> For vascular tissue regeneration, the potential of MSCs to differentiate toward vascular smooth muscle and endothelial lineages upon the effect of certain chemicals or mechanical factors and their ability to secrete certain chemical factors for paracrine influence are crucial. Differentiated MSCs and secreted



trophic factors combine to promote angiogenesis and arteriogenesis in injured tissues, thus restoring target organ function.

#### 4.1 | MSC-based therapy for myocardial infarction

Myocardial infarction is largely due to coronary artery diseases such as atherosclerosis, restenosis and plaque rupture, which results in the blockade of blood flow to cardiomyocytes. Current therapeutics have mainly focused on modulating hemodynamics, whereas more efforts need to be put into exploring therapeutics that will enhance cardiac regeneration, among which stem cell therapy emerges to be a promising choice.<sup>130</sup> MSCs are easily accessible and can differentiate into multiple cell types. In 2002, MSCs were shown to differentiate into cardiomyocytes *in vivo*, representing the basis of harnessing MSCs for cardiac regeneration in which massive cell replenishment is necessary.<sup>131</sup> However, limited engraftment of systematically administered MSCs at the infarct site and a lack of definite evidence demonstrating the differentiation of MSCs into cardiomyocytes has raised the recognition of paracrine mechanisms which contributes to improved cardiac function after MSC treatment.<sup>132</sup>

In the angiogenic process that is initiated after myocardial infarction, pericytes are first removed, followed by endothelial basement degradation. New matrix then forms and attracts endothelial cells. Vessel lumen is composed of endothelial cells as well as smooth muscle cells or pericytes recruited by the endothelial cells.<sup>133</sup> In contrast with the controversial evidence for differentiation ability of MSCs into cardiomyocytes, their differentiation ability to endothelial cells and smooth muscle cells or pericytes is better established.<sup>101,134,135</sup> Increased angiogenesis contributes to enhanced vascularity and therefore improved cardiac function.<sup>102</sup> The importance of paracrine mechanisms is addressed in experiments modifying the secretome of transplanted MSCs in preclinical studies.<sup>136,137</sup> Bioinformatic analysis revealed that MSCs may secrete factors that are involved in various physiological or pathological processes including neovascularization, inflammation, apoptosis, and cardiac remodeling.<sup>138</sup>

Factors that may influence the efficacy of MSC therapy include delivery method, timing of administration, and cell type.<sup>139</sup> Systemic administration of MSCs to model animals via intravenous injection was utilized initially but resulted in poor engraftment of the cells to infarct areas. Subsequently, local delivery methods such as intramyocardial, intracoronary, and transendocardial delivery were explored.<sup>140,141</sup> Intracoronary delivery may hamper coronary blood flow but is also associated with a higher remote engraftment rate.<sup>142</sup> Timing of stem cell administration is essential as inflammation after acute myocardial infarction can have an impact on the survival of stem cells, thus leading to a compromised effect.<sup>139</sup> However, no consistent conclusion has yet been achieved regarding the best time point for injections in animal studies, due to the difference in the length of studies and also the different ways to measure experiment outcomes. Bone marrow MSCs were the first to be applied in preclinical and clinical studies with the ease of their isolation and possibility of allogeneic cell therapy. Adipose tissue-derived MSCs emerged later as a promising cell source possessing similar properties with bone marrow MSCs and requiring a

less invasive isolation procedure. Recent studies showed that adipose tissue-derived MSCs exhibit greater potential as a therapeutic agent for myocardial regeneration.<sup>143,144</sup> Wharton's jelly-derived MSCs are younger stem cells and also displayed efficacy in treating acute myocardial infarction.<sup>145</sup> Before they can be clinically utilized as a therapeutic for myocardial infarction, safety of allogeneic and autologous MSC stem cell therapy must be closely examined.<sup>146,147</sup> Clinical trials conducted with results published in recent years are summarized in Table 2. Significant improvement was not achieved for all output measures and further optimization of MSC formulations is in need.

Until the mechanism for MSC driven boosting of cardiac regeneration is better elucidated, different approaches have to be investigated to prime MSCs to achieve more satisfying outcomes. It was first shown that hypoxia preconditioned MSCs enhanced regeneration of the infarct myocardium via trophic factors,<sup>148</sup> and with the aim of controlling the MSC secretome after transplantation, genetic manipulations were also harnessed. Overexpression of Akt in MSCs inhibits ventricular remodeling through increased secretion of angiogenic factors such as VEGF.<sup>122</sup> Angiogenic factor encoding genes can also be delivered directly to MSCs through non-viral methods.<sup>149</sup> Overexpression of HGF1 and SDF-1 in MSCs has been explored with the purpose of mobilizing bone marrow MSCs to the infarct site.<sup>150,151</sup> Yet more candidates for genetic manipulation need to be identified, which will require a more thorough understanding of the underlying mechanisms through which MSCs fulfill their function.

The trend for combining biomaterials with MSCs represents a promising new field for study. MSCs could be incorporated in biomaterials and controlled by them in terms of cell fate, secretion of trophic factors, and engraftment rate, thus improving their efficacy in cardiac repair.<sup>152</sup> Nanomaterials could serve as a non-viral method to deliver therapeutic factors which are essential for angiogenesis and stem cell recruitment.<sup>153,154</sup> Decellularized myocardium or injectable biopolymers may also serve as cell delivery platforms, which contribute to cell retention and facilitate heart repair.<sup>155-157</sup>

Current progress is being made in elucidating intercellular communication pathways through use of microparticles containing various factors including protein, mRNA and miRNAs. The therapeutic potential of microparticles derived for MSCs is starting to be recognized as they play a vital role in intercellular communication by signaling through receptors on target cells or the transfer of their contents.<sup>158</sup> MSC-derived exosomes, a type of MSC-derived microparticles, have been shown to exert anti-inflammatory, anti-apoptotic, and angiogenic effects.<sup>159-161</sup> Eventually, if mechanisms of MSC involvement in improved cardiovascular outcomes are better understood, present stem cell-based therapies may be replaced by cell-free therapy requiring only MSC-derived microparticles.

Although stem cell-based therapies in treating myocardial infarction are rapidly developing in recent years, challenges remain in both the pre-clinical areas such as the standardization of cell processing procedures and in clinical areas including selecting the best timing for cell transplant and the most suitable patients. Assessment of clinical potential is based on the robustness of efficacy in all tested animal models including at least one large animal model, and efficacy with the presence of co-morbidities

**TABLE 2** Published clinical trials utilizing MSC-based therapy to treat myocardial infarction

Clinical trials	Clinical condition	Intervention	Cell delivery route	Conclusion
NCT00768066 (TAC-HFT)	Ischemic cardiomyopathy	BMMSC injection vs placebo; BMC injection	Transendocardial	Transendocardial stem cell injection with MSCs or BMCs appeared to be safe.
NCT01087996 (POSEIDON)	Ischemic cardiomyopathy	Allogeneic BMMSC vs autologous BMMSC	Transendocardial	Allogeneic and autologous MSCs could both safely and efficiently induce structural and functional improvement.
NCT00587990 (PROMETHEUS)	Patients with chronic ischemic cardiomyopathy undergoing CABG	BMMSC injection vs CABG vs no intervention	Intramyocardial	Intramyocardial injection of autologous MSCs into akinetic yet non-revascularized segments produces comprehensive regional functional restitution
NCT01076920 (MESAMI 1)	Ischemic cardiomyopathy	BMMSC injection	Intramyocardial	Autologous MSCs can be safely administered to the patients' heart with chronic myocardial ischemia and may be associated with improvements in cardiac performance and LV remodeling.
NCT01291329	Acute ST-elevation myocardial infarction	WJMSCs vs placebo	Intracoronary infusion	Intracoronary infusion of WJMSCs is safe and effective in patients with AMI
NCT00677222	Acute ST-elevation myocardial infarction	MultiStem	Adventitial delivery	Adventitial delivery of MultiStem appears safe and delivery of $\geq 50$ million cells resulted in improved cardiac function
NCT00114452 (Prochymal)	Acute myocardial infarction	Placebo-controlled, dose-ranging allogeneic BMMSC	Intravenous	Intravenous allogeneic hMSCs are safe in patients after acute MI

BMMSC, bone marrow mesenchymal stem cell; BMC, bone marrow mononuclear cell; CABG, coronary artery bypass grafting; WJMSC, Wharton's jelly-derived mesenchymal stem cells.

and/or concomitant medication as recommended in the position paper by the European Society of Cardiology Working Group Cellular Biology of the Heart.<sup>162</sup> Moreover, multicenter, randomized controlled and double blinded studies should be undertaken to ultimately present MSC-based stem cell therapy as a potential routine clinical choice.

## 4.2 | MSC-based therapy for peripheral artery diseases

Peripheral artery diseases often result from occlusive artery diseases, typically atherosclerosis. They share risk factors with coronary artery diseases including hyperlipidemia, diabetes mellitus, and hypertension.<sup>163</sup> Chronic ischemia of peripheral tissues leads to serious conditions, including critical limb ischemia and amputation. Critical limb ischemia features objective arterial occlusion and manifests as pain even at rest or potential tissue death. Quality of life assessment in patients with critical limb ischemia showed comparable patterns with end-stage cancer patients.<sup>164</sup> The circulatory system can accommodate some changes such as in oxygen and nutrient needs due to the hemodynamic flexibility and some degree of cellular plasticity.<sup>165</sup> However, the blood flow requirement of end organs exceeds any self-modulating ability in ischemia.

Therapeutic attempts in restoring vascular function have mainly focused on revascularization, either with the induction of angiogenesis by growth factors and cytokines or with surgery. VEGF and FGF both induce endothelial cell proliferation and migration, thus forming new vessel branches from existing ones.<sup>166,167</sup> In response to shear stress change in the arterial stenosis, arterioles undergo a remodeling process,

as they do not have the capacity to create new arterial branches, featuring the degradation of extracellular matrix and formation of new three-layered arteries.<sup>168</sup> Although the utilization of proteins that target post-natal angiogenesis and arteriogenesis showed promising results, the intrinsic limit of their short half-life has hindered their long-term clinical benefit. Viral delivery of genes/plasmids *in vivo* aims to achieve long-term expression of the target protein, but these effects come at the expense of introducing viral infection. Another feasible therapeutic choice would be vascular surgery, however, patients' comorbidities or vascular state might hamper this possibility.

Bone marrow is the major reservoir of both hematopoietic stem cells and cells with angiogenic properties, given their similar origins. Mononuclear cells from the bone marrow can be isolated by density gradient centrifugation and demonstrate angiogenic potential in ischemic patients.<sup>169,170</sup> Compared to bone marrow mononuclear cells, MSCs possess a stronger capacity to differentiate toward smooth muscle cells and can be passaged *ex vivo* to largely reduce the bone marrow volumes required in clinical therapy.<sup>171,172</sup> In a randomized preclinical trial conducted by Lu et al., bone marrow MSCs were shown to be more effective than bone marrow mononuclear cells in improving limb perfusion in patients with diabetic critical limb ischemia and foot ulcers.<sup>172</sup> Adipose tissue-derived MSCs also exhibited angiogenic potential in animal studies,<sup>104,173</sup> by inducing endothelial differentiation<sup>114</sup> and secreting SDF-1<sup>174</sup> which can recruit endothelial progenitor cells from the bone marrow to ischemic sites.

The context of stem cell therapy is essential for maximizing the therapeutic efficacy. Adjuvant metabolic interventions such as

anti-oxidant and L-arginine therapy could improve neo-vascularization in animal studies and significantly reduce amputation rate in pilot MSC clinical trials.<sup>175,176</sup> Estrogen administration and exercise also exert positive effects in accelerating vascular repair, relying on their anti-apoptotic effect and increased VEGF secretion.<sup>177,178</sup>

Stem cell-based therapy has been widely explored in therapeutic angiogenesis and shows a great potential, however, challenges remain in selecting the best end point evaluations. Current physiological end points include ankle-brachial index and transcutaneous oxygen pressure, which are both non-invasive tests, but they mainly correlate with macrovascular function rather than microvascular function. Present imaging modalities such as ultrasound, magnetic resonance imaging and angiography, as well as functional outcomes such as pain-free walking rate and amputation free rate have also been investigated. Microcirculation imaging with optical coherence tomography could be utilized to define the microvascular function, representing a new means to evaluate therapeutic angiogenesis.<sup>179</sup>

### 4.3 | MSCs in vascular graft engineering

Cardiovascular diseases are the leading causes of medical problems all over the world, and often surgical bypass intervention is needed for treatment. One way to restore blood flow is to replace the injured or occluded blood vessels with engineered grafts. Saphenous veins and intercostal arteries are typical vascular graft candidates, but sometimes they are unavailable due to the severity of patients' conditions or they have been harvested in an earlier procedure. Successful application of synthetic grafts<sup>180</sup> or biologically based grafts<sup>181</sup> is limited by thrombosis, which may result in graft failure. As an alternative, tissue-engineered vascular grafts hold great promise in improving patients' outcomes, as they can be designed without these limitations.

Stem cells represent a potential cell source for vascular tissue engineering<sup>182</sup> including embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, and endothelial progenitor cells. SMCs and ECs derived from induced pluripotent stem cells have been shown to populate decellularized mouse aortic scaffolds to generate dual-seeding vessels. When such engineered vessels were grafted in vivo, they demonstrated better patency than cell-free scaffolds.<sup>183</sup> A recent study utilized embryonic stem cell-derived c-kit<sup>+</sup> cells as a source for SMCs and ECs and got encouraging results, with increased patency of in vivo grafted vessels for the generation of bioengineered vascular grafts.<sup>184</sup> However, the application of embryonic stem cells is largely hampered by ethical concerns and induced pluripotent stem cells require the transfection of pluripotency factors often via viral delivery. Endothelial progenitors and hematopoietic stem cells are additionally explored for their ability to differentiate toward endothelial cells and subsequent potential for vascular tissue engineering.<sup>185,186</sup> However, the scarcity of this cell source has limited their generalized use.

To engineer vascular grafts with mesenchymal stem cells, one choice is to seed undifferentiated cells onto scaffolds utilizing their cell recruiting abilities, and the other choice is to take advantage of the SMC and EC differentiation ability of MSCs in vitro and seed the partially differentiated cells onto scaffolds. A study published in 2007

showed that vascular grafts engineered by seeding undifferentiated human MSCs from the bone marrow onto nanofibrous scaffolds exhibited long-term patency compared to acellular grafts.<sup>187</sup> Furthermore, the vascular grafts acquired well-organized layers of ECs and SMCs which mimicked native vascular tissue after being transplanted in vivo. In 2009, Yilin Zhao et al. exploited the differentiation ability of MSCs to SMC and EC like cells, and by seeding them onto decellularized scaffolds, they managed to develop patent, anti-thrombogenic and mechanically stable vascular grafts after being transplanted in vivo.<sup>188</sup> Further efforts in optimizing tissue-engineered vascular grafts will allow for expansion of opportunities for clinical application.

## PERSPECTIVE

As MSCs are a promising cell type for vascular regeneration, it is important to overcome the challenges such as their heterogeneity and difficulties in ensuring their delivery and homing to injured tissue with high specificity. In addition, long-term safety data must be obtained before generalization of the therapy. In vivo tracking of MSCs with non-invasive techniques such as MRI and PET may contribute to our better understanding of their therapeutic effect. With sustained efforts to elucidate the mechanism of MSC involvement in vascular regeneration, MSC-based therapies hold great promise to generate a paradigm shift in regenerative medicine.

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## CONFLICT OF INTEREST

None declared.

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