Review Article

Potential involvement of human circulating CD14+ monocytes in tissue repair and regeneration

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Circulating CD14+ monocytes originate from hematopietic stem cells and are believed to be committed precursors for phagocytes such as macrophages. Recently, we have reported a primitive human cell population called monocyte-derived multipotential cells (MOMCs), which has a fibroblast-like morphology in culture and a unique phenotype positive for CD14, CD45, CD34, and type I collagen. MOMCs are derived from circulating CD14+ monocytes, but circulating precursors still remain undetermined. MOMCs contain progenitors with the capacity to differentiate into a variety of mesenchymal cells *in vitro*, including bone, cartilage, fat, and skeletal and smooth muscles. Moreover, MOMCs are able to differentiate into the cardiomyogenic and neuronal lineage by co-culturing with primary cultures of embryonic heart and brain, respectively. In addition, MOMCs have capacity of differentiating into endothelium of a mature phenotype with typical morphologic, phenotypic, and functional characteristics. *In vitro* generation of MOMCs from precursors within circulating monocytes requires their binding to fibronectin and exposure to soluble factors derived from activated platelets. In a rat model of cerebral ischemia, transplantation of MOMCs into the ischemic core results in a significant improvement in neurologic function, but this effect is primarily due to neovascularization through production of a large array of angiogenic factors by MOMCs with some contribution of their differentiation into mature endothelial cells. These findings indicate that circulating CD14+ monocytes are involved in a variety of physiologic functions other than innate and acquired immune responses, such as repair and regeneration of the damaged tissue, through a variety of mechanisms.

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Introduction

Circulating CD14+ monocytes originate from hematopoietic stem cells in the bone marrow and consist of 5 to 10% of circulating leukocytes in humans. They are a heterogeneous population in terms of surface markers, phagocytic capacity, and differentiation potentials, but are thought to be committed precursors in transit from the bone marrow to ultimate sites of activity. Circulating monocytes have capacity

to differentiate into a variety of phagocytes, including macrophages, dendritic cells, and osteoclasts. Until recently, it has been believed that the differentiation potential of monocytes was restricted to cells possessing phagocytic capacity, which function as phagocytes and antigen-presenting cells. However, recent accumulating evidence indicates that circulating monocytes have potential to differentiate into a variety of cell types other than phagocytes. In addition, monocytes are capable of producing various soluble factors that promote angiogenesis and tissue regeneration. Through these actions, it has been proposed that circulating $CD14^+$ monocytes contribute to tissue repair and regeneration. Our recent discovery of a primitive cell population termed monocyte-derived multipotential cells (MOMCs) supports this intriguing concept[1].

Identification of MOMCs

MOMCs, previously called monocyte-derived mesenchymal progenitors, are a human cultured cell population with a fibroblast-like morphology, and have a unique phenotype positive for CD14, CD45, CD34, and type I collagen[1]. These fibroblast-like cells efficiently made their appearance in cultures of peripheral blood mononuclear cells (PBMCs) for 7 to 10 days on fibronectin-coated plastic plates in medium supplemented with 10% fetal bovine serum as an only source of growth factors (Fig.1). Their unique shape was apparently different from the morphology of macrophages and immature dendritic cells, which are also derived from circulating $CD14^+$ monocytes. By ultrastructural examination by an electron microscope, MOMCs represented mixed features of phagocytes, mesenchymal cells, and endothelial cells. MOMCs expressed hematopoietic and monocyte lineage markers, including CD45, CD11b, CD14, and CD68. MOMCs also showed expression of several endothelial markers including CD34, VE-cadherin, and vascular endothelial growth factor (VEGF) receptor type 1 (VEGFR1). In addition, MOMCs are positive for type I and III collagens, fibronectin, and vimentin, which are typically produced by cells of mesenchymal origin. These findings clearly showed that MOMCs have mixed morphologic and phenotypic features of phagocytes, mesenchymal cells, and endothelial cells. Recent comparative analysis of gene expression profiles of MOMCs and other monocyte-derived cells, such as macrophages and dendritic cells using Affymetrix gene chips revealed that embryonic stem cell markers, Nanog and Oct-4, were exclusively expressed by MOMCs[2]. These unique characteristics are inconsistent with those of any other previously described cells derived from human peripheral blood.

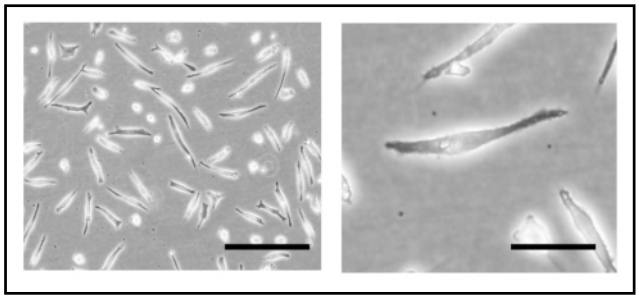

Fig.1 Morphology of MOMCs

MOMCs were generated by culturing PBMCs on fibronectin-coated plastic plates in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 7 days, and observed under an inverted microscope. Bars in left and right panels indicate 200 and 50 μm, respectively.

Appearance of MOMCs in cultures of PBMCs was completely inhibited by the depletion of $CD14^+$ monocytes, indicating circulating $CD14^+$ monocytes as the origin of MOMCs. To further confirm the monocytic origin of MOMCs, highly enriched $CD14^+$ monocytes were pre-labelled with a green fluorescent dye and cultured with unlabeled $CD14^-$ PBMCs on fibronectin-coated plates. As expected, fluorescence-labelled cells exclusively showed a fibroblast-like morphology and expressed CD34, indicating that precursors for MOMCs are present within circulating $CD14^+$ monocytes[1].

Mesenchymal differentiation potential of MOMCs

MOMCs have capacity to differentiate into a variety of mesenchymal cell types in specific permissive culture conditions principally developed for mesenchymal stem cells[1]. The induction treatment of MOMCs *in vitro* resulted in the expression of genes and proteins specific for bone, cartilage, fat, and skeletal muscle. MOMCs cultured in the condition medium prepared from dermal fibrobast cultures showed expression of α-smooth muscle actin, a marker for smooth muscle cells and myofibroblasts. The differentiation of MOMCs into individual mesenchymal cell types followed the steps observed in mesenchymal stem cell differentiation, in terms of the timing of lineage-specific transcription factor expression. For example, expression of the myogenic transcription factor MyoD preceded the expression of skeletal muscle actin and myosin.

Differentiation of MOMCs along the cardiomyogenic lin-

eage required a co-culture with cardiomyocytes prepared from the embryonic rat heart[3]. During the first 10 days of co-culture, the majority of MOMCs expressed the cardiomyocyte-specific transcription factors, such as Nkx2.5, GATA-4, and eHAND. After 2 weeks of the co-culture system, MOMCs gradually displayed a marked increase in surface area and became multiangular in morphology. Spontaneously beating MOMCs were observed at 3 weeks, but its efficiency was <5% of total MOMCs. These cells made contact with the surrounding rat cardiomyocytes and contracted in synchrony. At this stage, MOMCs expressed cardiomyocyte-specific structural proteins, such as α-sarcomeric actinin and troponin I, with typical staining patterns of the sarcomeric structures. In addition, MOMCs expressed connexin43, a protein consisting of gap junctions, and ultimately formed cell-to-cell contacts with the surrounding rat cardiomyocytes. Microinjection of the fluorescent dye into MOMCs revealed coupling as determined by direct dye transfer to neighboring rat cardiomyocytes. Cytoplasmic staining of atrial natriuretic peptide, which is almost exclusively secreted by atrial cardiomyocytes, was observed in the perinuclear regions. Expression of CD45 and CD14 was gradually down-regulated during cardiomyogenic differentiation and was eventually lost when they started spontaneous beating. An electrophysiological study revealed that contracting MOMCs showed spontaneous periodic action potentials typical of cardiac myocytes. These observations clearly indicate that a subset of MOMCs is able to differentiate into cardiomyocytes of a mature phenotype with typical electrophysiological characteristics *in vitro*.

Neuronal differentiation potential of MOMCs

Subsequently, we demonstrated that MOMCs were able to differentiate *in vitro* into the neuronal lineage using the similar co-culture assay using primary cultures of neuronal cells prepared from the embryonic rat brain[4]. Within 3 days of the co-culture, the majority of MOMCs showed nuclear expression of early neuroectodermal transcription factors, including Ngn2 and NeuroD. Over the next 2 weeks, a small population of MOMCs displayed a multi-polar neuron-like morphology. MOMCs expressing neurofilament had numerous axon-like processes projecting long distances and formed complex neural networks on the co-cultivated rat neurons. MOMCs expressed β3-tubulin and MAP2, which are known to be preferentially expressed by axonal processes in both shaft and spine synapses. In addition, these neuron-like MOMCs also exhibited nuclear expression of the neuron-specific RNA-binding protein Hu and the postmitotic neuron-specific nuclear protein NeuN. At this stage, MOMC-derived neuron-like cells lost the expression of CD45 and CD14. Again, the differentiation of MOMCs into neurons followed the steps observed in normal differentiation; i.e. the expression of pro-neuronal transcription factors preceded the expression of mature neuron-specific nuclear and structural proteins. Taken together, a subset of MOMCs is capable of differentiating along the neuronal lineage when placed into an appropriate environment, although their differentiation efficiency into mature neurons with typical morphologic and molecular features was very low (<5% of the total MOMCs). In the co-culture system, we could not exclude the possibility that cell fusion was partly responsible for the phenotypic change of MOMCs.

Endothelial differentiation potential of MOMCs

We discovered that MOMCs are able to also differentiate into endothelium of a mature phenotype with typical morphologic, phenotypic, and functional characteristics[5]. MOMCs treated with a combination of angiogenic growth factors for 7 days changed their morphology from spindle-shaped to caudate, and these cells had numerous rod-shaped microtubulated structures resembling Weibel-Palade bodies. Almost every MOMC expressed endothelial markers, such as CD31, CD144, VE-cadherin, VEGFR1, VEGFR2, Tie-2, von Willebrand factor, endothelial nitric oxide synthase, and CD146, but expression of CD14 and CD45 was markedly down-regulated. Functional characteristics, including vWF release upon histamine stimulation and up-regulated expression of VEGF and VEGFR1 in response to hypoxia, were indistinguishable between the MOMC-derived endothelial-like cells and cultured human umbilical vein endothelial cells. In contrast to low differentiation efficiency to bone, fat, skeletal and cardiac muscle, and neural lineages, morphologic and molecular features typical of mature endothelial cells were observed in nearly all adherent MOMCs in cultures. MOMCs responded to angiogenic stimuli and promoted the formation of mature endothelial cell tubules in Matrigel® cultures. Finally, in xenogenic transplantation studies using a severe combined immunodeficient mouse model in which syngeneic colon carcinoma cells were injected subcutaneously with or without human MOMCs, co-transplantation of

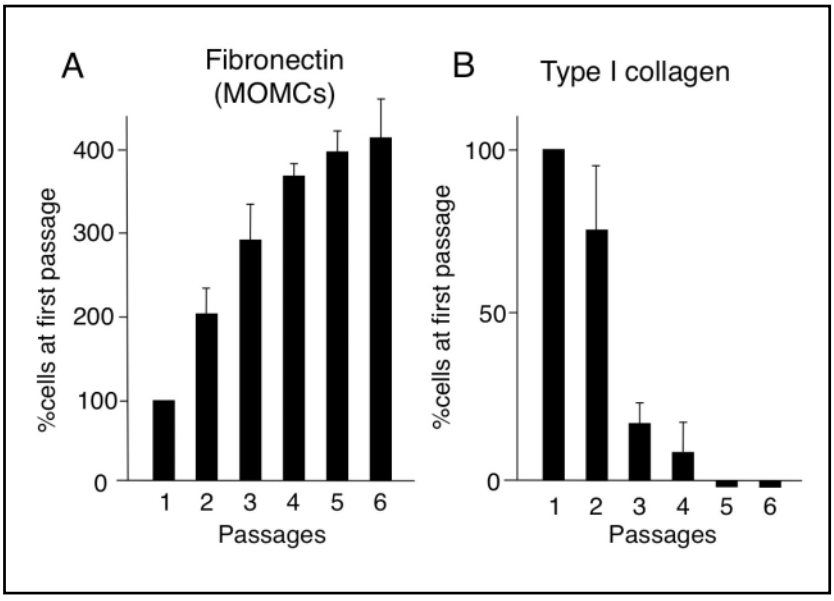

Fig.2 Proliferative capacity of adherent cells obtained in MOMC generation cultures on fibronectin or type I collagen

PBMCs were cultured on plastic plates coated with fibronectin (A) or type I collagen (B). The medium containing floating cells was exchanged with fresh medium every 3 days. At day 7, all adherent cells were detached, counted, and replated on plates coated with fibronectin or type I collagen. Results are expressed as proportion of the number of adherent cells recovered at each passage to the number of adherent cells recovered at the first passage. The mean and standard deviation of 5 independent experiments are shown.

MOMCs significantly promoted the formation of blood vessels, and more than 40% of the tumor vessel sections incorporated human endothelial cells derived from MOMCs. These findings together indicate that MOMCs can differentiate along the endothelial lineage in a specific permissive environment *in vitro* and *in vivo*. However, in the tumor neovascularization model, only 10% of transplanted MOMCs differentiated *in vivo* into mature endothelial cells incorporating into the vessel wall, and the majority of transplanted MOMCs remained $CD45^+CD34^+$type I $collagen^+$ fibroblast-like cells. This suggests an idea that MOMCs promote blood vessel formation not only through vasculogenesis but also through angiogenesis. In fact, MOMCs were capable of producing a large amount of angiogenic factors, including VEGF, basic fibroblast growth factor (bFGF), and hepatocyte-growth factor (HGF).

Phagocytic cell differentiation potential of MOMC

Interestingly, MOMCs still retain potentials to differentiate into phagocytes. Upon exposure to macrophage-colony stimulating factor (M-CSF), MOMCs displayed a marked increase in surface area, and represented phagocytic capacity. These features are comparable to those of macrophages. However, $CD4^+$ T cells exposed to antigen-captured MOMCs resulted in loss of proliferation and cytokine production upon subsequent antigenic stimulation, which are consistent with a state of T-cell anergy. This indicates that MOMCs may exert antigen-specific immune regulatory function, which is potentially useful in suppression of harmful immune responses, such as autoimmunity and transplantation rejection.

In addition, MOMCs cultured on fibronectin at high density resulted in the appearance of tartrate-resistant acid phosphatase-positive giant multi-nucleated cells forming actin-ring[6]. A subset of these cells showed bone resorption capacity on dentine slices and expression of genes for cathepsin K and calcitonin receptor, characteristic of functional osteoclasts. MOMCs expressed receptor activator of nuclear factor-κB ligand (RANKL), which is required for osteoclast formation from mononuclear precursors. These results indicate that human MOMCs are able to express RANKL and differentiate into functional osteoclasts without RANKL-expressing accessory cells, such as osteoblasts and activated T cells.

Molecular factors required for MOMC generation

In vitro generation of MOMCs was not observed when circulating $CD14^+$ monocytes were cultured alone on fibronectin-coated plastic plates. On the other hand, MOMCs were not generated in cultures of PBMCs on plastic plates without fibronectin coating. This clearly indicates that circulating $CD14^-$ cells and binding to fibronectin are both required for the MOMC generation from precursors within circulating $CD14^+$ monocytes[1]. MOMCs were efficiently generated when

CD14+ monocytes were cultured alone on fibronectin in the conditioned medium generated by culture of circulating CD14- cells on fibronectin, suggesting an important role of soluble factor(s) produced by CD14- cells, rather than a cell-to-cell contact. In this regard, we have recently found that soluble factors and microparticles released from activated platelets promote the MOMC generation. Adherent cells with spindle-shaped morphology were also obtained when we set up MOMC cultures on type I collagen, instead of fibronectin. These spindle-shaped cells expressed CD14 and type I collagen, but not CD34. Moreover, proliferation capacity was different between adherent cells generated on fibronectin and type I collagen (Fig.2). The number of MOMCs generated on fibronectin increased during cultures on fibronectin up to five passages. In contrast, the cells generated on type I collagen did not have the ability to proliferate or *in vitro* differentiation potential into bone, cartilage, fat, or endothelium. These findings together indicate that binding of CD14+ monocytes to fibronectin in a critical step for generation of MOMCs. In this regard, generation of MOMCs in cultures of circulating CD14+ monocytes on fibronectin was completely inhibited by addition of anti-α5-integrin neutralizing antibody or a synthetic peptide that competed with the RGD domain of fibronectin. In summary, generation of MOMCs from circulating CD14+ monocytes requires an interaction between the RGD domain of fibronectin and cell-surface $\alpha_5\beta_1$ integrin, as well as soluble factors and microparticles released from activated platelets.

CD14+ MOMC precursors in circulation

It is obvious that precursors for MOMCs reside within circulating CD14+ monocytes, but we still have not identified specific markers for the MOMC precursors. Until now, several distinct human cell populations that originate from circulating CD14+ monocytes and have capacity to differentiate into non-phagocytes have been described. Zhao and colleagues demonstrated that pluripotent stem cells were generated from a subset of peripheral blood monocytes by repeated stimulation with a high concentration of M-CSF and phorbol myristate acetate[7]. These spindle-shaped CD34+ cells termed pluripotent stem cells (PSCs) had the capacity to differentiate along several distinct cell lineages, including macrophages, T cells, epithelial cells, endothelial cells, neuronal cells, and hepatocytes. On the other hand, monocyte-derived endothelial progenitor cells (EPCs) resided within the CD14+CD34low cell population were shown to have ability to differentiate not only into endothelial cells, but also into osteoblasts, adipocytes, or neuronal cells[8]. Finally, fibrocytes were reported as a circulating cell population with fibroblast properties that plays an important role both in normal wound repair and in pathological fibrotic responses[9]. Fibrocytes were characterized by its distinctive phenotype positive for CD45, CD34, and type I collagen, and a characteristic chemokine receptor expression pattern CCR3+CCR5+CCR7+CXCR4+ was shown to be useful in identifying fibrocytes in circulation[10]. Fibrocytes were shown to lack the expression of CD14 in the original report[9], but recent studies have found that fibrocytes derive from circulating CD14+ monocytes[11].

These monocyte-derived cells commonly have spindle-shaped morphology and express both CD45 and CD34, but have several distinct characteristics. For example, PSCs and fibrocytes are able to self-replicate and expand in long-term cultures, whereas MOMCs have limited lifespan like monocytic EPCs. Since cellular origins of these cell types have not been fully identified yet, circulating precursors within circulating CD14+ monocytes may be different. Alternatively, distinct differentiation potentials of these primitive cells might be due to different culture conditions of the same precursors.

Roles of MOMCs in tissue repair and regeneration

Circulating CD14+ monocytes are long believed to be committed precursors specific for phagocytes, but there are several lines of evidence showing that circulating CD14+ monocytes have the potential to differentiate into various non-phagocytes, including mesodermal and neuroectodermal lineages. These observations challenge the traditional view of the biology of the monocyte/macrophage system. Our recent findings on MOMCs indicate that circulating monocytes contain a cell population that has the capacity to differentiate into several distinct cell lineages through differentiation into MOMCs at least *in vitro*. Efficiencies of differentiation in *in vitro* cultures are greatly variable among cell lineages: i.e. high efficiency for endothelial differentiation, but low efficiency for differentiation into skeletal and cardiac myogenic lineages and neuronal lineage. Recently, we have successfully generated a rat counterpart of MOMCs using the method used for generation of human MOMCs. The adherent cells obtained in these cultures were comparable to human MOMCs in terms of their morphology and expression of CD45, CD11b, CD34, and type I collagen. We have recently examined *in*

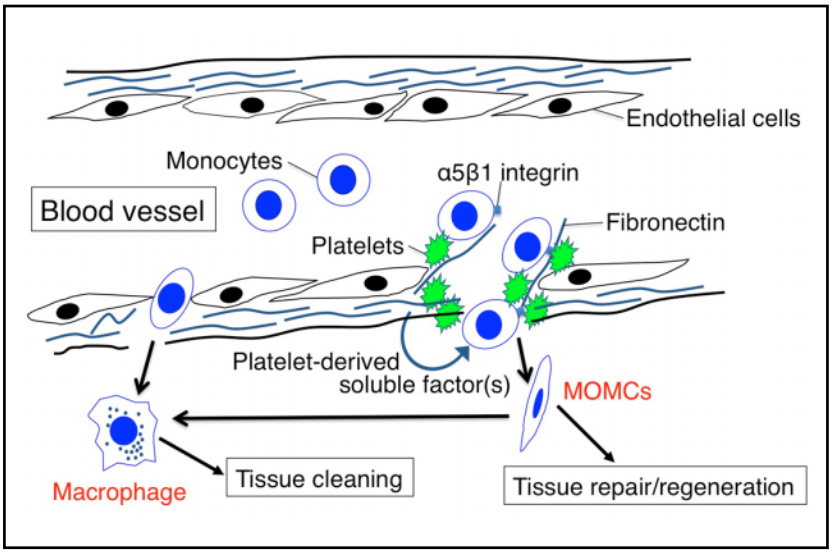

Fig.3 Potential physiological roles of circulating CD14⁺ monocytes in tissue repair and regeneration

Upon tissue damage, CD14⁺ monocytes in circulation move to the extra-vascular site of injury and are differentiated into macrophages. On the other hand, circulating monocytic precursors differentiate into MOMCs upon exposure to external signals and subsequently into tissue-specific cells in response to organ-specific cues provided by the surrounding environment. In addition, MOMCs are able to release various soluble mediators, including angiogenic factors and chemokines. Tissue infiltrating cells derived from circulating CD14⁺ monocytes collaborate each other on promotion of tissue repair and regeneration.

vivo differentiation potentials of MOMCs by delivering rat MOMCs directly into the damaged tissue of syngeneic rats[12]. In a rat model of cerebral ischemia, transplantation of rat MOMCs into the ischemic core resulted in a significant improvement in neurologic function, but transplantation of macrophages did not. This improvement is mediated primarily through neovasculization at the ischemic boundary. We evaluated the fate of the transplanted MOMCs in the ischemic brain using MOMCs derived from syngeneic GFP-transgenic rats for cell transplantation. Despite multipotent differentiation potentials of human MOMCs *in vitro*, only <10% of viable MOMCs at 4 weeks after transplantation differentiated into mature endothelial cells and contributed to the blood vessel formation. There was no NeuN⁺ neuronal cell derived from rat MOMCs, and the majority of transplanted rat MOMCs remained CD45⁺CD34⁺type I collagen⁺ fibroblastic cells. On the other hand, human MOMCs produce a large variety of cytokines and growth factors that promote angiogenesis and tissue repair, including VEGF, HGF, basic FGF, Ang-1, placenta growth factor (PlGF), and stromal cell-derived factor-1 (SDF-1)[5, 12]. Thus, neovascularization through production of a large array of angiogenic factors in combination with *in vivo* differentiation into mature endothelial cells appears to be a primary mechanism for MOMC-mediated functional recovery in this animal model. It is possible that *in vivo* differentiation from MOMCs to non-phagocytic cells may not be a common event even in case of massive tissue injury, but further analyses using different animal models are necessary to draw a conclusion.

A series of researches on MOMCs will lead to further progress in the understanding of the differentiation potential of circulating CD14⁺ monocytes and the roles they play in the physiologic state. Figure 3 illustrates potential physiological roles of circulating CD14⁺ monocytes in tissue repair and regeneration. Differentiation from circulating CD14⁺ monocytes to non-phagocytic cells may not be induced during normal development, but may be readily induced in the presence of cues, such as massive tissue injury. Upon tissue damage, monocytes in circulation move to the extra-vascular site of injury, and are exposed to inflammatory cytokines and differentiate into macrophages, which take up debris to clean the tissue and induce T-cell responses. On the other hand, since the differentiation of CD14⁺ monocytic precursors into MOMCs requires binding to fibronectin and soluble factor(s) from activated platelets, circulating monocytic precursors may encounter these signals at the site of tissue injury and inflammation. Then, these external signals would induce specific intracellular signals in the infiltrating monocytic precursors, resulting in modulation of the expression profiles of transcription factors, which induce the their differentiation into MOMCs. MOMCs subsequently differentiate into tissue-specific cells in response to organ-specific cues provided by the surrounding environment. Macrophages derived from MOMCs may suppress excessive immune responses by their regulatory capacity. In addition, MOMCs are able to release various soluble mediators, including angiogenic factors and chemokines, thereby promoting angiogenesis and tissue regeneration. Therefore, strategies to recruit MOMCs to the

site of injury may be a useful approach for repairing the damaged tissue. However, further *in vivo* studies evaluating whether MOMCs promote regeneration in the damaged tissues are necessary to confirm their potential use in cell therapy.

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