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Mesenchymal Stem Cells Are Recruited into Wounded Skin and Contribute to Wound Repair by Transdifferentiation into Multiple Skin Cell Type

Mikako Sasaki,1,2 Riichiro Abe,2 Yasuyuki Fujita, Satomi Ando, Daisuke Inokuma, and Hiroshi Shimizu3

Mesenchymal stem cells (MSCs) can differentiate not only into mesenchymal lineage cells but also into various other cell lineages. As MSCs can easily be isolated from bone marrow, they can be used in various tissue engineering strategies. In this study, we assessed whether MSCs can differentiate into multiple skin cell types including keratinocytes and contribute to wound repair. First, we found keratin 14-positive cells, presumed to be keratinocytes that transdifferentiated from MSCs in vitro. Next, we assessed whether MSCs can transdifferentiate into multiple skin cell types in vivo. At sites of mouse wounds that had been i.v. injected with MSCs derived from GFP transgenic mice, we detected GFP-positive cells associated with specific markers for keratinocytes, endothelial cells, and pericytes. Because MSCs are predominantly located in bone marrow, we investigated the main MSC recruitment mechanism. MSCs expressed several chemokine receptors; especially CCR7, which is a receptor of SLC/CCL21, that enhanced MSC migration. Finally, MSC-injected mice underwent rapid wound repaired. Furthermore, intradermal injection of SLC/CCL21 increased the migration of MSCs, which resulted in an even greater acceleration of wound repair. Taken together, we have demonstrated that MSCs contribute to wound repair via processes involving MSCs differentiation various cell components of the skin. The Journal of Immunology, 2008, 180: 2581–2587.

Bone marrow has an extremely complex cellular organization of bone marrow stroma, to maintain the hematopoietic microenvironment. Other than hemopoietic stem cells and differentiated lineages, bone marrow contains a subset of nonhemopoietic cells, mesenchymal stem cells (MSCs) that account for roughly 0.01–0.001% of the bone marrow derived cell population (1). These rare, heterogeneous cells have the capacity to proliferate and differentiate into mesenchymal lineage cells such as osteoblasts, adipocytes, and chondrocytes (1, 2) (3, 4). Thus, MSCs are thought to be the key in maintaining the bone marrow microenvironment. Various mesenchymal tissues such as s.c. fat also contain MSCs (5).

Recent reports show that MSCs may have the ability to differentiate into other lineage cells in vitro, such as endothelial cells (6, 7), neural cells (8, 9) and hepatocytes (10, 11). In vivo studies have also shown that MSCs can differentiate into tissue-specific cells in response to cues provided by different organs (12).

In addition to pluripotency, MSCs are known to have immunosuppressive effects involving various mechanisms, resulting in evading the allogeneic host immunosurveillance system (13). Therefore, recent studies have suggested that MSCs are promising candidates for cell-based tissue engineering, to repair or replace important damaged tissues (14) such as after myocardial infarction (15), and spinal injury (16). However, there have been no investigations into whether the introduction of MSCs into skin wounds is effective or not.

So far, MSCs have already been used in several clinical trials including neurological diseases and spinal injury (17, 18), with results that have fallen short of any high expectations. It has been speculated that one of reasons was an insufficient knowledge of physiological behavior of MSCs. The detailed mechanisms of specific cell type differentiation from MSCs still remain to be identified. To better handle this potentially useful cell type and provide further promising novel regenerative cell therapies, we urgently require a much greater in-depth knowledge of MSCs to make better use of them in therapies.

We hypothesize that induction of mechanical stress in skin results in the release of various cytokines, especially chemokines which recruit blood-circulating MSCs (19). At the same time, these chemokines increase bone marrow stem cell mobility, thereby, facilitating MSCs mobilization into the peripheral blood and into sites of wound healing. Accumulating MSCs at wounded sites are able to transdifferentiate into multiple skin component cell types, thus contributing to wound repair.

In this study, we cultured MSCs in various culture medium, and have identified certain conditions under which MSCs efficiently differentiate into keratinocytes in vitro. Additionally, we have i.v. injected MSCs into wounded mice, and have investigated whether

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4 Abbreviations used in this paper: MSC, mesenchymal stem cell; FISH, fluorescence in situ hybridization; SMA, smooth muscle actin; TARC, thymus and activation regulated chemokine; MIP, macrophage inflammatory protein; SLC, secondary lymphoid tissue chemokine; CTACK, cutaneous T cell-attracting chemokine; HPF, high power field.

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MSCs migrate and become engrafted into wounded skin to promote wound healing.

Materials and Methods
Isolation and culture of MSCs from mouse bone marrow
Bone marrow-derived cells were collected by flushing the femurs and tibias from C57BL/6 and GFP-transgenic (under control of β-actin promoter) male mice (The Jackson Laboratory). These cells were cultured in MesenCult basal medium containing MSC stimulatory supplements (StemCell Technologies). After 48 h, the nonadherent cells were removed and fresh medium was added to the cells. Medium was changed every 2 or 3 days. The adherent spindle-shaped cells were further propagated for three passages.

Flow cytometry
Cultured MSCs were analyzed by flow cytometry (FACS Calibur; BD Biosciences). Cells were incubated with anti-CD31, CD34, CD44, CD90 (BD Biosciences), CD29 (Cymbus Biotechnology), and cytokeratin 14 (Chemicon) and secondary FITC-conjugated Abs (The Jackson Laboratory).

Differentiation culture of MSCs for mesenchymal lineage
MSCs were placed in basic medium, consisting DMEM (Invitrogen Life Technologies), 10% FBS, 1% penicillin, 1% streptomycin, 1% amphotericin B, and then specific supplements for mesenchymal lineage differentiation were added (20). Adipogenic differentiation was induced by basic medium with 0.5 μM dexamethazone, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.1 mM indomethacin (Sigma-Aldrich) (21). Osteogenic differentiation was achieved by basic medium containing 0.1 μM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate (Sigma-Aldrich) (22). Chondrogenic differentiation was induced by basic medium containing 50 μM ascorbic acid, 0.1 μM dexamethasone, 10 ng/ml TGF-β (R&D Systems), 40 μg/ml t-proline (Sigma-Aldrich), and 100 μg/ml sodium pyruvate (Wako) (23). Each specific differentiation medium was changed every 2–3 days. Confirmation of differentiation of the cells to adipocytes, osteocytes and chondrocytes were performed by staining with oil red O, Von Kossa, and toluidine blue, respectively.

Induction of MSC into keratinocyte differentiation
MSCs were plated into 8-well slide glass chamber and cultured in keratinocyte basal medium (Invitrogen Life Technologies) containing 0.5 nM bone morphogenetic protein-4 (BMP-4) (R&D Systems), 0.3 mM ascorbic acid, 0.5 μg/ml hydrocortisone or 3 nm glucose human epithelium growth factor (Cambrex). After 7 days culture, MSCs were stained with cytokeratin 14 Abs (Chemicon).

Intravenous injection of MSCs into the wounded mice
All animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol. Female C57BL/6 mice were anesthetized and 10-mm full thickness punch biopsy wounds were made. One million MSCs derived from male GFP transgenic mice were injected into the tail vein of back skin-injured mice. All wounds were repaired within 2 wk. When the wound was repaired, the area skin was collected and analyzed. Furthermore, wound area was measured in mice with and without MSC injection.

To calculate migrated MSCs in wound skin, wound sites were removed 3 days later and examined for the presence of GFP + MSCs by quantitative flow cytometric analysis following proteolytic digestion. For quantitative flow cytometric analysis, excised skin (250 mg biopsy/animal) was chopped into small fragments, then incubated for 1 h at 37°C in RPMI 1640 containing 10% FBS, 2 mg/ml collagenase, and 20 mg/ml DNase I. The resulting single-cell suspension was examined by flow cytometry to determine the number of fluorescent fibrocytes present.

Fibroblasts (1 × 10⁸) cultured from adult GFP transgenic mouse skin were injected into the tail vein of back skin-injured mice. After 8 days, wound sites were removed to analyze.

Immunofluorescence staining
Skin sections were stained with anti-GFP Ab (Molecular Probes). In addition, skin sections were treated with primary Abs against CD45, CD31, pan-cytokeratin (Frogen), α-smooth muscle actin (SMA; LAB VISION), and CCR7 (Santa Cruz Biotechnology). Secondary Abs conjugated to rhodamine-isothiocyanate (Southern Biotechnology) were used for fluorescence staining detection together with a confocal laser scanning fluorescence microscope (FV1000; Olympus).

Results
Characterization of isolated MSCs
Cell surface markers were assessed using flow cytometry to characterize isolated MSCs. MSCs expressed CD29, CD44, and CD90, but not CD34 and CD31 (Fig. 1A) consistent with previous reports (22, 24). MSCs were further characterized by confirming their ability to undergo specific adipogenic, osteogenic, and chondrogenic differentiation. These cells were positive for oil red O staining, Von Kossa’s staining, and toluidine blue staining, indicating adipogenic, osteogenic, and chondrogenic respective cell type differentiation (Fig. 1, B–D). Only cells that met these criteria were used in subsequent experiments.
and Y chromosomes using FISH methods. If MSCs and recipient result of spontaneous cell fusion, we analyzed the presence of X positive monocytes/macrophages (CD11b positive) were also GFP-positive adipocytes were found. In addition, very few GFP-positive macrophages were also detected (1.5/HPF and 2.4%), no (27) were differentiated from injected MSCs. Although GFP-positive macrophages were colocalized with pan-cytokeratin (Fig. 2, Ad–f), CD31 (endothelial cell marker, Fig. 2, Ad–f), and α-SMA (myofibroblast and pericyte marker; Fig. 2, Ag–i). The number of GFP-positive, pan-cytokeratin-positive cells is 1.0/HPF and the percentage of GFP-positive in all pan-cytokeratin-positive cells is 0.14% (Table I). The percentage of keratin 14-positive cells at the beginning of culture, as bone marrow cells. Enhancement of keratinocyte commitment (0.48%) was clearly observed when 0.5 nM BMP-4 was added for 7 days (Fig. 1F).

MSCs differentiate into multiple skin cell types

Recent reports have shown that MSCs can differentiate into various cell types. In skin cells, endothelial cells, pericytes, monocytes/macrophage, and adipocytes have been reported (24, 25). However, it is currently unknown whether MSCs can differentiate into keratinocytes. For that reason, we assessed whether MSCs can differentiate into keratinocytes in vitro. MSCs were exposed to 0.5 nM BMP-4 at different days of culture. Keratin 14 positive cells were presumed to identify MSC transdifferentiated keratinocytes. There were no keratin 14-positive cells at the beginning of culture, as bone marrow cells. Enhancement of keratinocyte commitment (0.48%) was clearly observed when 0.5 nM BMP-4 was added for 7 days (Fig. 1F).

Cultured MSCs express keratin14

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MSCs migrate in response to specific chemokine gradients

Several papers have reported (28, 29) that MSCs constitutively express various chemokine receptors such as CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6. If chemokine/chemokine receptor interactions contribute to the recruitment of MSCs to damaged tissues, a specific chemokine should be up-regulated in the target tissue together with a partner receptor expressed on the MSCs. The ability of MSCs to migrate in response to chemotactic signals was investigated using a chemotaxis assay.

Chemokine receptor expression on MSCs were examined to determine potential migratory reaction to stimuli. MSCs expressed skin cells were fused, these cells would contain XXXY chromosomes. Although we analyzed in total ~1 × 10^4 cells, we detected no GFP-positive cells containing XXXY chromosomes. All GFP positive cells contain XY chromosomes (Fig. 2B) indicating that the incidence of MSCs and skin cell fusion is an extremely rare event.

MSCs in wound site. A, MSC differentiate into multiple components of the skin. GFP positive cells (green) were colocalized with pan-cytokeratin (red), (d–f) CD31 (red), and (g–i) α-SMA (red). Nuclear staining (c and f) and CD31 (l) are blue. These data suggests MSCs were differentiated into keratinocytes, endothelial cells, and pericytes, respectively. B, Detection of X and Y chromosomes using FISH methods. All GFP positive cells contains XY chromosomes. Arrow (blue), Y; arrow head (red), X.
MSCs are recruited by specific chemokin/chemokine receptor interactions. A, Chemokine receptor expression on MSCs were analyzed by flow cytometry. Staining with a specific Ab for each chemokine receptor (solid line) and the background staining with the nonspecific Ig Ab (negative isotype matched control; shaded profile). B, Chemotaxis assays were undertaken in vitro. MSCs were added to the upper well of a 8-μm pore Transwell chamber. Indicated recombinant chemokines were added to the upper and/or lower plate. MSCs migration rates increased in response to medium containing recombinant SLC/CCL21 or TARC (∗, p < 0.05) vs medium alone (n = 4). C, Migrated MSCs induced by SLC/CCL21 in chemotaxis assay were positive of CCR7 expression. D, In wound site of MSC injected mice, GFP positive cells (green) were colocalized with CCR7 (red). Nuclear staining was blue.

**Table I. MSC differentiated into various cell component of the skin**

<table>
<thead>
<tr>
<th>Differentiated MScs/HPF (×40)</th>
<th>MSC-Derived, Specific Cell Marker + Cell (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocyte</td>
<td>1.0 (14/2828)</td>
</tr>
<tr>
<td>Endothelial cell</td>
<td>4.7 (13.2/2182)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>1.5 (2.4/3129)</td>
</tr>
<tr>
<td>Pericyte</td>
<td>0.2 (3.3/15)</td>
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SLC/CCL21 specifically led to the accumulation of MSCs in wound skin and accelerated MSCs-induced wound healing

We analyzed the number of injected MSC (1 × 10⁶) which accumulated in the skin wound. Using flow cytometry, 7.4 × 10⁵ MSC were detected in the wound skin at 3 day after wound, and tend to decrease (Fig. 4A). To assess the ability of MSC recruitment by these chemokines in vivo, we intradermally injected these chemokines to the periphery of wounded skin in MSCs injected mice. The number of GFP-positive cells in the wound sites was then calculated, when the wound was repaired (each group, n = 5). Although TARC failed to influence the number of MSCs compared with controls, SLC/CCL21 significantly increased the number of GFP-positive MSCs in wounded skin (Fig. 4B). Time course analysis of the number of MSCs (2, 6, and 12 wk) showed the number of MSCs were gradually decreased (Fig. 4C). The quality of the wound was not significant difference between healed skin of MSC injected mice and that of control mice at 15 days (Fig. 4D). In addition, the number of GFP⁺ endothelial cells showed a trend to increase, and the number of GFP⁺ pericytes increased significantly compared with control (Fig. 4E). We therefore surmised that SLC/CCL21 was capable of attracting MSCs, which participated in the host skin angiogenic wound response.

Furthermore, to evaluate the contribution of MSCs in reducing the wound area at 8 days, we measured wound size. Quantification of wound size demonstrated only 9.3 mm² in MSCs injected-mice, compared with 23 mm² in control mice (Fig. 4F). Wound size in the mice with fibroblasts injection was not significantly different from those of control mice (data not shown). MSCs injected-mice repaired wounds faster than control mice. In addition, i.v. injection of MSCs and intradermal injection of SLC/CCL21 together further encouraged wound repair. These data suggest that MSCs contribute to wound repair by differentiating into multiple skin cell types. Furthermore, SLC accelerated wound closure of MSCs injected-mice in a dose-dependent manner (Fig. 4G).

We showed that circulating MSC was recruited by SLC/CCL21. Furthermore, SLC/CCL21 accelerated MSCs accumulation in wound site, especially the formation endothelial transdifferentiated cells. For that reason, we speculated that SLC/CCL21 may enhance differentiation of MSCs into endothelial cells. To investigate endothelial differentiation of MSC was enhanced by SLC/CCL21, we cultured MSCs in endothelial cell differentiation medium containing SLC/CCL21. CD31 positive cells were presumed to identify MSC transdifferentiated endothelial cells. The number of endothelial cell was no different between healed skin of SLC/CCL21 added and not added (data not shown).
After full-thickness cutaneous injury, MSCs have been vigorously investigated, but it has not attained a consensus whether MSCs can contribute significantly to regenerate wounded tissue. Although the transdifferentiation mechanism of MSCs has been investigated, it has not attained a sufficient level that can be used in clinical applications. Accumulation of circulating MSCs, predominantly delivered from bone marrow stroma to the specific tissue might be one of the efficient strategies for tissue regeneration. In our study, 7.4 × 10^7 MSCs were detected in the wound skin of MSC injected mouse (1 × 10^6 i.v.). Recent paper (32) reported that injected MSCs (1 × 10^6 i.v.) were detected predominantly in blood (5 × 10^4) and lungs (5 × 10^4) and relatively low numbers of MSCs were detected in femoral bone marrow (1 × 10^5), spleen (1 × 10^5), liver (2 × 10^5), and brain (5 × 10^5). These data indicate that the transplanted MSCs circulate in the blood and are capable of extravasating into tissue. It seems to be reasonable that 6.9 × 10^6 MSC were detected in 1 cm wounded skin in our experiment (1 × 10^7 MSC were injected).

There are still questions about origin and multipotentiality of MSCs. MSCs can be considered nonhemopoietic multipotent stem-like cells that are capable of differentiating into both mesenchymal and nonmesenchymal lineages (33). However, there is no specific single marker to clearly define MSCs. In fact, at present, MSCs are identified through a combination of physical, phenotypic, and functional properties. The classical assay used to identify MSCs is the colony forming unit assay that identifies adherent spindle shaped cells that proliferate to form colonies and can be induced to differentiate into adipocytes, osteocytes, chondrocytes (33). Because MSC in our study qualify this criteria, we use the term "mesenchymal stem cells" in this article. Furthermore, it is still questionable whether MSCs from bone marrow differentiate into keratinocytes in normal wound repair. From present data, we showed that injected-MSCs contribute to wound repair via accumulation in wound site. In addition, it has been reported that MSCs circulate in normal state (19). However, it is difficult to label resident MSC because there is no specific single marker to clearly define MSCs. Further studies should prove MSCs have true stem cell potential.

If the marker for the keratinocyte-transdifferentiating MSCs is found, we can enrich them by the marker and transdifferentiate them into keratinocytes. There are still questions about origin and multipotentiality of MSCs. MSCs can be considered nonhemopoietic multipotent stem-like cells that are capable of differentiating into both mesenchymal and nonmesenchymal lineages (33). However, there is no specific single marker to clearly define MSCs. In fact, at present, MSCs are identified through a combination of physical, phenotypic, and functional properties. The classical assay used to identify MSCs is the colony forming unit assay that identifies adherent spindle shaped cells that proliferate to form colonies and can be induced to differentiate into adipocytes, osteocytes, and chondrocytes (33). Because MSC in our study qualify this criteria, we use the term "mesenchymal stem cells" in this article. Furthermore, it is still questionable whether MSCs from bone marrow differentiate into keratinocytes in normal wound repair. From present data, we showed that injected-MSCs contribute to wound repair via accumulation in wound site. In addition, it has been reported that MSCs circulate in normal state (19). However, it is difficult to label resident MSC because there is no specific single marker to clearly define MSCs. Further studies should prove MSCs have true stem cell potential.

**Discussion**

In this article, we showed that MSCs may come to express keratin 14, keratinocyte marker, in vitro. In wounds, we also showed that MSCs have the capacity to differentiate into multiple skin cell types including keratinocytes, endothelial cells, pericytes, and monocytes. Furthermore, circulating MSC recruitment was induced by a specific chemokine (SLC/CCL21)chemokine receptor (CCR7) interaction both in vitro and in vivo. Intradermal injection of SLC/CCL21 significantly accelerated wound closure by increasing rates of MSC accumulation, especially the formation of endothelial transdifferentiated cells.

In wound healing process, inflammation is very important phenomenon because inflammation process including induction of inflammatory factors and accumulation of various inflammatory cells. Inflammatory factors and inflammatory cells start tissue regeneration by replenishment of cells and extracellular components. We previously reported that SLC/CCL21 was expressed in keratinocytes of wounded skin (31). Taken together, stimulated keratinocytes produce SLC/CCL21 and MSCs are accumulated in wound site, then contribute wound repair by transdifferentiation into multiple skin cell types.

Several clinical trials using MSCs have been attempted, including for the treatment of neurological diseases (17), spinal injury (16), and myocardial infarction (15). Although several reports have proved some efficacy for MSCs, it is still controversial whether MSCs can contribute significantly to regenerate damaged tissue via tissue specific transdifferentiation. This may be explained, at least in part, by poor viability of the transplanted cells. Furthermore, a suitable microenvironment to promote specific transdifferentiation might be strictly provided, so that MSCs local application without additional treatment failed to form a biologically complete tissue. Physiological accumulation of enough MSCs might induce further cell type differentiation, resulting in better functional organization of the wounded tissue. Although the transdifferentiation mechanism of MSCs has been vigorously investigated, it has not attained a sufficient level that can be used in clinical applications. Accumulation of circulating MSCs, predominantly delivered from bone marrow stroma to the specific tissue might be one of the efficient strategies for tissue regeneration. In our study, 7.4 × 10^7 MSC were detected in the wound skin of MSC injected mouse (1 × 10^6 i.v.). Recent paper (32) reported that injected MSCs (1 × 10^6 i.v.) were detected predominantly in blood (5 × 10^4) and lungs (5 × 10^4) and relatively low numbers of MSCs were detected in femoral bone marrow (1 × 10^5), spleen (1 × 10^5), liver (2 × 10^5), and brain (5 × 10^5). These data indicate that transplanted MSCs circulate in the blood and are capable of extravasating into tissue. It seems to be reasonable that 6.9 × 10^6 MSC were detected in 1 cm wounded skin in our experiment (1 × 10^7 MSC were injected).

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If the marker for the keratinocyte-transdifferentiating MSCs is found, we can enrich them by the marker and transdifferentiate them into keratinocytes.
keratinocyte from MSC easily. However, unfortunately there is no report about it. In our study, we showed BMP-4 induced keratinocyte differentiation in vitro, suggesting the receptor of BMP-4 or other related protein may be related to keratinocyte differentiation.

In this study, we showed that a specific chemokine may recruit circulating MSCs into the wound site, resulting in the stimulation of wound repair via the promotion of angiogenesis. Our findings indicate that MSCs together with tissue specific chemokines might be more effectively used for clinical applications.

Stem cells in bone marrow include hemopoietic stem cells, MSCs, and multipotent adult progenitor cells (12). It is still unknown whether hemopoietic stem cells are able to transdifferentiate into nonhemopoietic cells. Conversely, some specific environments, reported as niche microenvironments, are required to transdifferentiate into several organ-specific cells from bone marrow stem cells. In the skin, several reports showed there are a number of bone marrow cells that traffic through skin (34). Wounding stimulated the engraftment of bone marrow cells to the skin and induced bone marrow-derived cells to incorporate into and differentiate into nonhemopoietic skin structures. Although there are numerous reports of tissue-specific transdifferentiation from bone marrow, evidence has not suggested that direct transdifferentiation form bone marrow to specific tissue cells contributes to tissue regeneration. This also includesMSCs transdifferentiation. Other explanations of the effect of bone marrow application might be bone marrow cell-derived soluble factors, which regulate inflammation and angiogenesis. Recently, we reported that a specific chemokine, CTACK, is the major regulator involved in the migration of keratinocyte precursor cells from bone marrow into skin (31). Furthermore, increased bone marrow-derived keratinocyte migration by CTACK significantly accelerated the skin wound healing process. Because we demonstrated that MSCs migrate into wounded skin via SLC/CCL21-CCR7, it is interesting that MSCs (CD34− and bone marrow-derived keratinocyte precursor cells (CD34+), which have a different phenotype, recruit and transdifferentiate into keratinocytes by different chemokine systems. In addition, chemokines induce wound repair via the accumulation of MSCs and bone marrow-derived keratinocyte precursors.

Finally, several clinical trials using MSCs have been attempted, including for spinal injury and myocardial infarction, which are difficult to heal by normal tissue regeneration. And it has been reported that MSCs application is very effective for these diseases. Therefore, we expect that MSCs therapy also accelerates skin wound healing especially refractory, common therapy-resistant skin ulcer.

Taken together, specific chemokine/chemokine receptor interactions involving stem cells are promising therapeutic candidates to regulate the regeneration phenomenon.

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Disclosures
The authors have no financial conflict of interest.

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