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# Mesenchymal Stem Cells Are Recruited into Wounded Skin and Contribute to Wound Repair by Transdifferentiation into Multiple Skin Cell Type<sup>1</sup>

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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.

**B**one marrow has an extremely complex cellular arrangement of bone marrow stroma, to maintain the hemopoietic microenvironment. Other than hemopoietic stem cells and differentiated lineages, bone marrow contains a subset of nonhemopoietic cells, mesenchymal stem cells (MSCs)<sup>4</sup> 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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<sup>4</sup> 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  $\beta$ -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  $\mu$ M 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  $\mu$ M dexamethazone, 50  $\mu$ M ascorbic acid, and 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich) (22). Chondrogenic differentiation was induced by basic medium containing 50  $\mu$ M ascorbic acid, 0.1  $\mu$ M dexamethazone, 10 ng/ml TGF- $\beta$  (R&D Systems), 40  $\mu$ g/ml L-proline (Sigma-Aldrich), and 100  $\mu$ g/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  $\mu$ g/ml hydrocortisone or 3 ng/ml 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<sup>+</sup> 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 ( $1 \times 10^6$ ) 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 (Progen),  $\alpha$ -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).

### Detection of X- and Y-chromosomes by fluorescence in situ hybridization (FISH) analysis

To investigate whether the GFP positive cells in the skin of MSC injected mice were the result of transdifferentiation or cell fusion with host cells, we used MSCs of a different sex from the recipient mice, and investigated the tissue using FISH analysis. X- and Y-chromosomes were detected using the Dual Color Detection kit (Cambio) according to the manufacturer's protocol (Cy5 for Y chromosomes and Cy3 for X chromosomes) and immediately viewed with a confocal laser scanning fluorescence microscope.

### Chemokine receptor expression in MSCs

Total RNA was isolated from MSCs. RT-PCR analysis of mRNA from chemokine receptors and GAPDH were performed in a thermocycler (GeneAmp PCR system 9600; PerkinElmer). Primers are as follows: CXCR4 (sense: 5'-ACTGCATCATCATCTCCAAGC-3', antisense: 5'-CTCTCGAAGTACATCCTTGC-3'); CCR4 (sense: 5'-TCTACAGCGGCATCTTCTTCAT-3', antisense: 5'-CAGTACGTGTGGTTGTGCTCT-3'); CCR5 (sense: 5'-CTGGCCATCTCTGACCTGTTTTTC-3', antisense: 5'-CAGCCTGTGCCTTCTTCTCAT-3'); CCR7 (sense: 5'-ACAGCGGCCTCAGAAAGAGAGCGC-3', antisense: 5'-TGACGTATAGGCAATGTTGAGCTG-3'); CCR10 (sense: 5'-GGCCCTGACTTGCCTTTG-3', antisense: 5'-GCTGCCAGTAGATCGGCTGT-3'); GAPDH (sense: 5'-GAGGGCCATCCACAGTCTTC-3', antisense: 5'-CATCACCATCTTCCAGGAGCG-3').

MSCs were incubated with anti-CXCR4, CCR4, CCR5 (BD Pharmingen), CCR7 (Santa Cruz Biotechnology), and CCR10 (Calbiochem) and then analyzed by flow cytometry as described above.

### Chemotaxis assay

MSCs migration was evaluated using a Chemotaxicell (Kurabo) according to the manufacturer's instructions. The contents of the upper and lower chambers were separated by polycarbonate filter (8- $\mu$ m pore size). MSCs were resuspended at  $1.0 \times 10^5/100 \mu$ l in Mesencult basal medium and seeded in the upper chamber. Recombinant monokine induced by IFN- $\gamma$ , stromal derived factor 1, thymus and activation regulated chemokine (TARC), macrophage inflammatory protein (MIP)-3 $\alpha$ , secondary lymphoid tissue chemokine (SLC/CCL21) and cutaneous T cell-attracting chemokine (CTACK) (R&D Systems) were used as chemoattractants in the lower chamber. The chambers were incubated overnight at 37°C. Results are expressed as number of migrated cells in lower chamber. To confirm CCR7 expression, migrated MSC in SLC/CCL21 chemotaxis assay were stained with CCR7 Ab (Santa Cruz Biotechnology).

### Wound healing analysis

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 immediately after injury. Subsequently, the chemokine (SLC/CCL21, TARC, or CTACK; a total of 3  $\mu$ g in 100  $\mu$ l) or PBS (100  $\mu$ l), as vehicle-only control, was intradermally injected into the periphery of the wounds. Standardized images of wounds were recorded using a digital camera for analysis of daily wound sizes.

### Induction of MSC into endothelial cell differentiation

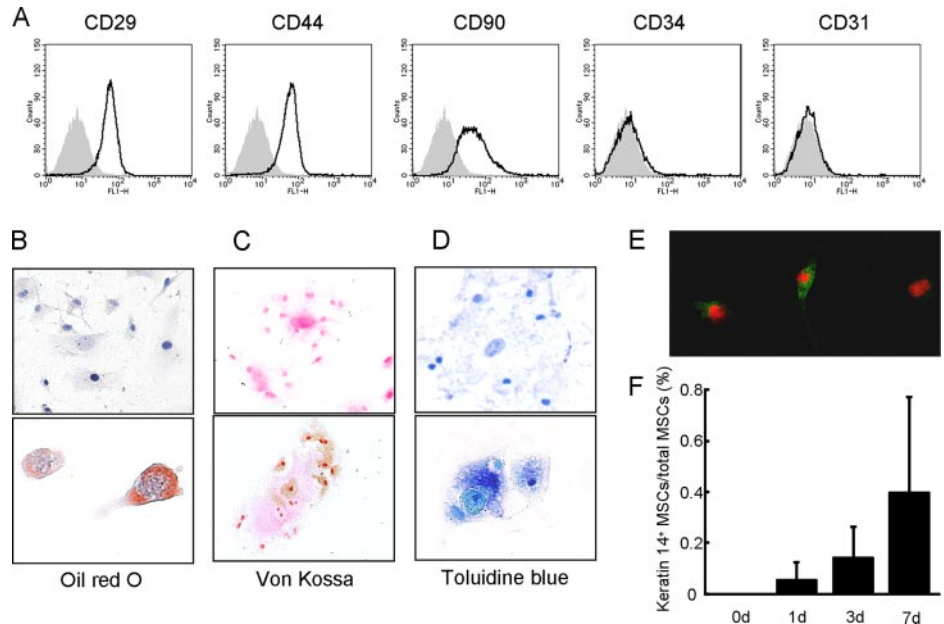
MSCs were plated into fibronectin coated 8-well slide glass chamber and cultured in DMEM containing with 50 ng/ml VEGF, 10 ng/ml bFGF, 3% FBS and 0, 1, 10, or 100 ng/ml SLC/CCL21. After 7 days culture, MSCs into endothelial cell differentiation were evaluated by CD31 Immunofluorescence staining.

## 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.

**FIGURE 1.** MSCs have a potential to differentiate into keratinocyte. *A*, Cell surface markers of MSCs were assessed using FACS. MSCs expressed CD29, CD44, and CD90, but not CD34, CD31. *B*, Adipogenic differentiation was revealed with oil red O staining. *C*, Osteogenic differentiation was confirmed by Von Kossa's staining. *D*, The chondrogenic potential of MSCs was determined by staining for toluidine blue. In *B–D*, upper panels are before differentiation induction, and lower panels are after differentiation induction, representatively. To investigate whether MSCs differentiate into keratinocytes, we cultured MSCs in various condition medium. *E*, MSCs cultured for 7 days were stained with cytokeratin 14 (green). Red fluorescence is nuclear staining by propidium iodide. *F*, The percentage of keratin 14 + MSCs in total MSCs at 0, 1, 3, and 7 day keratinocyte differentiation culture.



#### Cultured MSCs express keratin14

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. 1*F*).

#### MSCs differentiate into multiple skin cell types

Recent reports (22) have shown that MSCs were mobilized and differentiate into cardiomyocytes after myocardial infarction. Furthermore transplanted MSCs improved cardiac function in dilated cardiomyopathy (26). For that reason, we speculated that MSCs may differentiate into multiple skin cell types during wound healing. GFP transgenic mouse-derived MSCs were injected i.v. into injured mice. When wound repaired, the area skin was collected and performed immunofluorescence staining. GFP positive cells were colocalized with pan-cytokeratin (Fig. 2, *Aa–c*), CD31 (endothelial cell marker, Fig. 2, *Ad–f*), and  $\alpha$ -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 number of MSCs that differentiated into endothelial cells was 4.7/HPF, and 13.2% of endothelial cells were GFP positive. In particular, 33.0% of pericytes (recognized as  $\alpha$ -SMA-positive, CD31-negative and located close to the capillary (27)) were differentiated from injected MSCs. Although GFP-positive macrophages were also detected (1.5/HPF and 2.4%), no GFP-positive adipocytes were found. In addition, very few GFP-positive monocytes/macrophages (CD11b positive) were also found (data not shown).

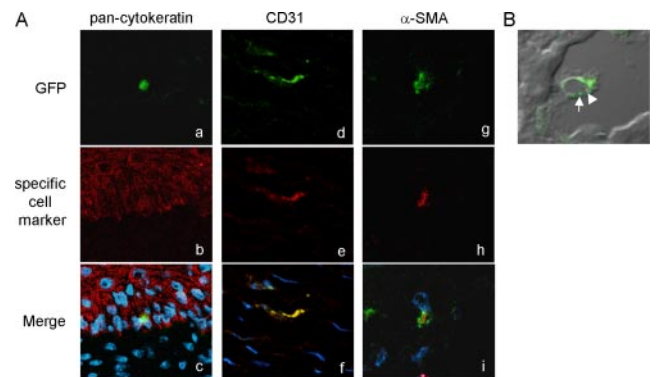
To exclude the possibility that skin cell differentiation was the result of spontaneous cell fusion, we analyzed the presence of X and Y chromosomes using FISH methods. If MSCs and recipient

skin cells were fused, these cells would contain XXXY chromosomes. Although we analyzed in total  $\sim 1 \times 10^4$  cells, we detected no GFP-positive cells containing XXXY chromosomes. All GFP positive cells contain XY chromosomes (Fig. 2*B*) indicating that the incidence of MSCs and skin cell fusion is an extremely rare event.

#### 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



**FIGURE 2.** MSCs transdifferentiate into multiple skin cell type in wound site. *A*, MSC differentiate into multiple components of the skin. GFP positive cells (green) were colocalized with (*a–c*) pan-cytokeratin (red), (*d–f*) CD31 (red), and (*g–i*)  $\alpha$ -SMA (red). Nuclear staining (*c* and *f*) and CD31 (*i*) 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.

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

	Number of Differentiated MSCs/HPF ( $\times 40$ )	MSC-Derived, Specific Cell Marker <sup>+</sup> Cell (%) (Number of GFP <sup>+</sup> , Cell Marker <sup>+</sup> /Number of Cell Marker <sup>+</sup> )
Keratinocyte	1.0	0.14 (4/2828)
Endothelial cell	4.7	13.2 (24/182)
Macrophage	1.5	2.4 (3/129)
Pericyte	0.2	33.0 (5/15)

the transcripts for CCR4, CCR5, CCR7, and CCR10. Transcripts for other chemokine receptors were not detected. Protein expression was determined by flow cytometry analysis. CCR4, CCR5, CCR7, CCR10, and CXCR4 were also expressed in 14.3, 19.9, 10.0, and 38.0% of MSCs, respectively (Fig. 3A). These data are consistent with previous report that showed a minority of MSCs (2–25%) were expressed as a restricted set of chemokine receptors (29, 30).

To confirm that these receptors were actually functional in these cells, *in vitro* chemotaxis assays were undertaken. The following ligand-receptor combinations were investigated: TARC for CCR4, MIP-3 $\alpha$  for CCR5, SLC/CCL21 for CCR7, and CTACK for CCR10. Neither CTACK nor MIP-3 $\alpha$  enhanced MSC migration. In contrast, SLC/CCL21 and TARC induced MSCs' migration in a dose-dependent manner (Fig. 3B). Indeed, migrated MSCs induced by SLC/CCL21 in chemotaxis assay were almost all positive of CCR7 expression (Fig. 3C). Furthermore, in wound site of MSC injected mice, injected MSCs (GFP positive) expressed CCR7 (Fig. 3D).

#### SLC/CCL21 specifically led to the accumulation of MSCs in wounded skin and accelerated MSCs-induced wound healing

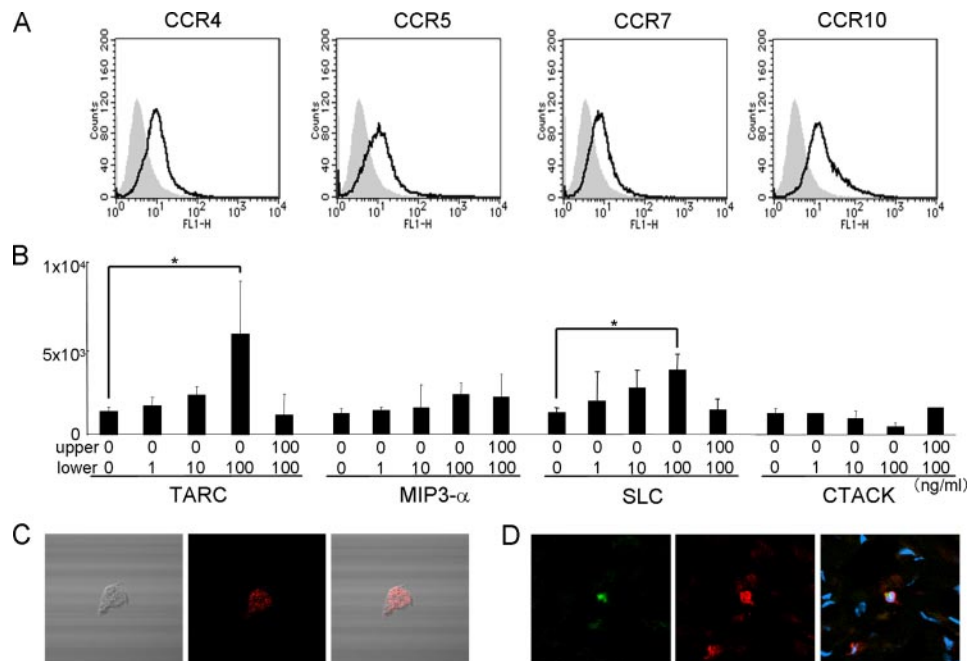
We analyzed the number of injected MSC ( $1 \times 10^6$ ) which accumulated in the skin wound. Using flow cytometry,  $7.4 \times 10^2$  MSC were detected in the wound skin at 3 day after wound, and tend to decrease (Fig. 4A). To assess the ability of MSC recruitment by these chemokines *in vivo*, we intradermally injected these chemo-

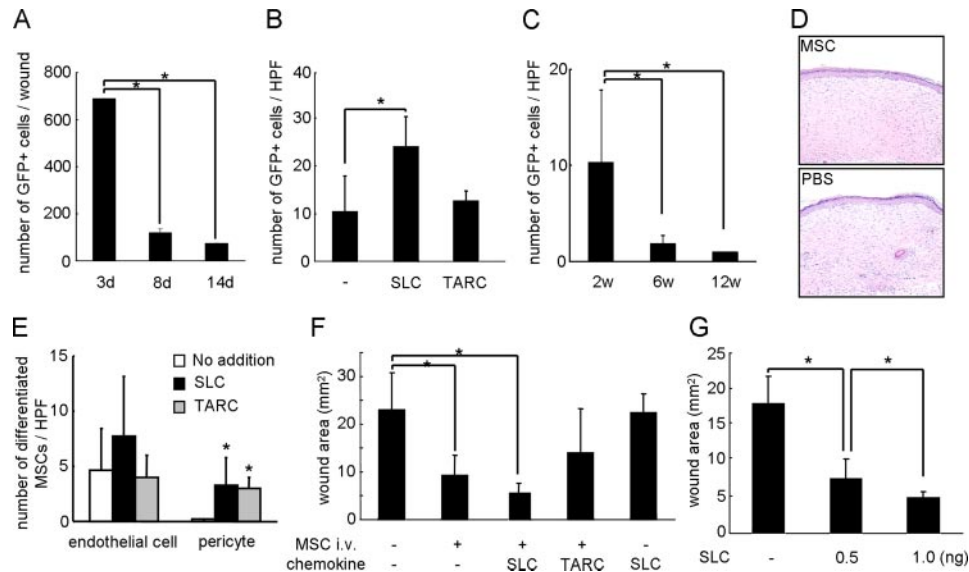
kinines to the periphery of wounded skin in MSCs injected mice. The number of GFP-positive cells in the wound sites was then calculated, when the wound was repaired (each group,  $n = 5$ ). Although TARC failed to influence the number of MSCs compared with controls, SLC/CCL21 significantly increased 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<sup>+</sup> endothelial cells showed a trend to increase, and the number of GFP<sup>+</sup> 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 mm<sup>2</sup> in MSCs injected-mice, compared with 23 mm<sup>2</sup> 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 difference between SLC/CCL21 added and not added (data not shown).

**FIGURE 3.** MSCs are recruited by specific chemokine/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- $\mu$ m 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.





**FIGURE 4.** MSCs are recruited into wounded skin and contribute to wound repair. *A*, The number of MSCs accumulated into wounded sites was analyzed by flow cytometry (\*,  $p < 0.05$ ). *B*, The number of MSCs in wounded sites with local application of 3  $\mu\text{g}$  of chemokine (SLC/CCL21 or TARC) was counted in the HPF (\*,  $p < 0.05$ ). Time course analysis of the number of MSCs (2, 6, and 12 wk) (*C*), and the number of MSC-differentiated endothelial cells and pericytes (*E*) were also counted (\*,  $p < 0.05$ ). *D*, H&E staining of healed skin of MSC injected mice and that of PBS-injected mice at 15 days after full-thickness cutaneous injury. *F*, Wound size was measured at 8 days after injury and subsequent chemokine treatment (total 3  $\mu\text{g}$  in 100  $\mu\text{l}$ ) or PBS (100  $\mu\text{l}$ ) as control (5 mice in each group). Intradermal injection of SLC/CCL21 significantly accelerated wound closure (\*,  $p < 0.05$ ). *G*, SLC accelerated wound closure of MSCs injected-mice in a dose-dependent manner (\*,  $p < 0.05$ ).

## 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 strategy for tissue regeneration. In our study,  $7.4 \times 10^2$  MSC were detected in the wound skin of MSC injected mouse ( $1 \times 10^6$  i.v.). Recent paper (32) reported that injected MSCs ( $1 \times 10^6$  i.v.) were detected predominantly in blood ( $5 \times 10^4$ ) and lungs ( $5 \times 10^4$ ) and relatively low numbers of MSCs were detected in femoral bone marrow ( $1 \times 10^2$ ), spleen ( $1 \times 10^4$ ), liver ( $2 \times 10^3$ ), and brain ( $5 \times 10^2$ ). These data indicate that the transplanted MSCs circulate in the blood and are capable of extravasating into tissue. It seems to be reasonable that  $6.9 \times 10^2$  MSC were detected in  $1 \times 1$  cm wounded skin in our experiment ( $1 \times 10^6$  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, and chondrocytes (33). Because MSC in our study qualify this criteria, we used 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 has true stem cell potential.

If the marker for the keratinocyte-transdifferentiating MSCs is found, we can enrich them by the marker and transdifferentiate

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 from bone marrow to specific tissue cells contributes to tissue regeneration. This also includes MSCs 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<sup>-</sup> and bone marrow-derived keratinocyte precursor cells (CD34<sup>+</sup>), 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

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