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Published January 19, 2015, doi:10.4049/jimmunol.1400914

Transplanted Bone Marrow–Derived Circulating PDGFRα$^+$ Cells Restore Type VII Collagen in Recessive Dystrophic Epidermolysis Bullosa Mouse Skin Graft

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Recessive dystrophic epidermolysis bullosa (RDEB) is an intractable genetic blistering skin disease in which the epithelial structure easily separates from the underlying dermis because of genetic loss of functional type VII collagen (Col7) in the cutaneous basement membrane zone. Recent studies have demonstrated that allogeneic bone marrow transplantation (BMT) ameliorates the skin blistering phenotype of RDEB patients by restoring Col7. However, the exact therapeutic mechanism of BMT in RDEB remains unclear. In this study, we investigated the roles of transplanted bone marrow–derived circulating mesenchymal cells in RDEB (Col7-null) mice. In wild-type mice with prior GFP-BMT after lethal irradiation, lineage-negative/GFP-positive (Lin$^-$/GFP$^+$) cells, including platelet-derived growth factor receptor α-positive (PDGFRα$^+$) mesenchymal cells, specifically migrated to skin grafts from RDEB mice and expressed Col7. Vascular endothelial cells and follicular keratinocytes in the deep dermis of the skin grafts expressed SDF-1α, and the bone marrow–derived PDGFRα$^+$ cells expressed CXCR4 on their surface. Systemic administration of the CXCR4 antagonist AMD3100 markedly decreased the migration of bone marrow–derived PDGFRα$^+$ cells into the skin graft, resulting in persistent epidermal detachment with massive necrosis and inflammation in the skin graft of RDEB mice; without AMD3100 administration, Col7 was significantly supplemented to ameliorate the pathogenic blistering phenotype. Collectively, these data suggest that the SDF1α/CXCR4 signaling axis induces transplanted bone marrow–derived circulating PDGFRα$^+$ mesenchymal cells to migrate and supply functional Col7 to regenerate RDEB skin. *The Journal of Immunology*, **2015, 194: 000–000.**

Recessive dystrophic epidermolysis bullosa (RDEB) is a severe genetic blistering skin disease in which mutations in both alleles of the type VII collagen gene (COL7A1) abrogate functional expression of Col7, which physiologically secures the attachment of epidermis to the underlying dermis in the cutaneous basement membrane zone. Previously, we reported that allogeneic BMT in the circulation of fetal RDEB mice could restore functional Col7 in the cutaneous basement membrane zone after birth, thereby improving the blistering phenotype of the skin and extending survival (1). Furthermore, in a clinical trial, allogeneic BMT in human RDEB patients ameliorated their fragile skin condition by enhancing Col7 expression (2). However, the exact mechanism underlying the BMT-mediated Col7 supplementation in RDEB skin is still unknown.

Bone marrow contains at least two different lineages of cells: hematopoietic and mesenchymal cells. Hematopoietic cells are generated from hematopoietic stem cells (HSCs), which reside in the bone marrow stem cell niche. Mesenchymal cells are thought to be derived from mesenchymal stem cells (MSCs) in the bone marrow, although the definitive nature of MSCs is still under investigation (3, 4). MSCs were originally defined as stem cells that could differentiate into mesenchymal lineages, such as osteocytes, chondrocytes, and adipocytes, in culture (5–8). However, MSCs were also shown to differentiate into other lineages, including neuronal and epithelial cells (9, 10).

In the field of skin regeneration, bone marrow has been shown to provide inflammatory and noninflammatory cells, including mesenchymal fibroblasts and epidermal keratinocytes, to wounded areas (11–13). We previously reported that bone marrow–derived platelet-derived growth factor receptor α (PDGFRα)-positive mesenchymal cells play a crucial role in regenerating the engrafted skin of wild-type mice and RDEB mice by providing bone marrow–derived fibroblasts and keratinocytes (14). Although

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Received for publication April 10, 2014. Accepted for publication December 12, 2014.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a Health and Labour Sciences Research Grant (for Research of Intractable Diseases and for Clinical Trial on Development of New Drugs and Medical Devices in 2014) from the Ministry of Health, Labour, and Welfare of Japan.

S.I., E.A., and R.F. designed the research, performed the research, and analyzed the data. T.C., Y.K., J.A.M., and H.I. analyzed the data. S.I., E.A., R.F. and K.T. wrote the manuscript. Y.K., J.U., J.K., and M.I. contributed vital reagents. K.T. and Y.K. were responsible for the final approval of the manuscript.

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Abbreviations used in this article: BMT, bone marrow transplantation; HMGB1, high mobility group box 1; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; PDGFRα, platelet-derived growth factor receptor α; RDEB, recessive dystrophic epidermolysis bullosa; SDF-1α, stromal cell–derived factor 1α.

PDGFRα is known to be expressed by cutaneous mesenchymal cells such as dermal fibroblasts and follicular papilla cells, the appearance of PDGFRα$^+$ bone marrow cell-derived keratinocytes is consistent with previous reports that the PDGFRα$^+$ cell population in bone marrow contains ectodermally derived MSCs with neural and epithelial differentiation capacity (15, 16).

Regarding the homing of marrow-derived nonhematopoietic cells into the area in need of repair, previous studies demonstrated that various stimuli derived from injured tissues mobilize MSCs from the bone marrow to accelerate tissue repair (17, 18); however, circulating MSCs are relatively rare under physiologic conditions (19, 20). We also previously demonstrated that necrotic skin, including detached RDEB epithelia, releases high mobility group box 1 (HMGB1), which then mobilizes PDGFRα$^+$ bone marrow cells into the circulation. However, the mechanisms by which bone marrow–derived mesenchymal cells home to injured skin and the role of these cells in RDEB skin after BMT have not been elucidated.

Among chemokines and their receptors, the C-X-C type chemokine ligand 12 (CXCL12), known as stromal cell-derived factor 1α (SDF-1α), and its receptor, CXCR4, have been documented to direct the migration of stem/progenitor cells to various tissues (21–25). In bone marrow, endothelial cells and stromal cells in the HSC niche express SDF-1α, which acts as a chemoattractant for HSCs and supports the survival and proliferation of HSCs via CXCR4 signaling (25, 26). SDF-1α is also implicated in the migration of circulating CXCR4$^+$ stem/progenitor cells to damaged tissues (21–24). The SDF-1α–dependent homing mechanism of circulating endothelial progenitor cells to infarcted myocardium is well established (23, 24). We also previously reported that circulating osteoblast progenitor cells migrate to bone-forming sites via SDF-1α–mediated chemoattraction (22). Furthermore, it has been reported that culture-expanded MSCs are recruited to bone fracture sites by the SDF-1α/CXCR4 pathway after systemic injection (21). SDF-1α expression is regulated by the transcription factor hypoxia inducible factor-1 in endothelial cells in ischemic tissue, thus enabling CXCR4$^+$ stem/progenitor cells in the circulation to target ischemic or injured tissue (23). Although these reports clearly illustrate the SDF-1α/CXCR4 axis as a pivotal mechanism for recruiting various types of bone marrow–derived cells to injured tissues, the roles and functions of these cells in tissue regeneration have not been fully elucidated.

In this study, we examined the role of migrating, bone marrow-derived PDGFRα$^+$ cells in restoring Col7 in RDEB mouse skin engrafted onto GFP-BMT mice. We then investigated the involvement of the SDF-1α/CXCR4 axis in the migration of circulating bone marrow–derived PDGFRα$^+$ cells into the engrafted mouse skin to ameliorate the RDEB phenotype.

Materials and Methods

Mice

All animal experiments were performed according to the guidelines of the Ethical Committee for Animal Experiments of Osaka University Graduate School of Medicine. All experimental mice were housed in cages with a 12-h light-dark cycle. Solid food and water were supplied ad libitum. C57BL/6N mice were purchased from CLEA Japan (Tokyo, Japan). Type VII collagen (Col7) heterozygous ($^{+/-}$) mice were crossed to breed Col7-null ($^{-/-}$) mice, which phenotypically mimic several conditions, including extensive cutaneous blistering suggestive of the human RDEB phenotype (27). C57BL/6N mice that ubiquitously expressed enhanced GFP (GFP, referred to as GFP mice) were provided by Masaru Okabe (Osaka University). SDF-1α/GFP knock-in mice, in which the *GFP* gene was inserted into the SDF-1α locus, were provided by Masaru Ishii (Osaka University).

Bone marrow transplantation

Bone marrow cells were isolated from 6-wk-old male GFP mice by flushing the tibiae and femurs. The recipients were 6-wk-old female C57BL/6N mice that were lethally irradiated with 10 Gy of X-rays, and each irradiated recipient received 5×10^6 bone marrow cells from GFP mice. Experiments were performed on the BMT mice at least 6 wk after BMT.

Skin graft model

Full-thickness skin from wild-type and Col7-null newborn mice (2 × 2 cm) was carefully isolated by excision after the mice had been euthanized under systemic anesthesia and engrafted on the backs of GFP-BMT mice and wild-type mice just above the muscular fascia. The wound sites on the skin-grafted mice were then covered with bandaging tape to protect the grafted skin from scratching until further examination.

ELISA for SDF-1α

Peripheral blood was taken from the heart using a 22-gauge needle and a 1-ml syringe containing heparin. For the preparation of serum, whole blood was centrifuged at 1200 × *g* for 15 min at 4°C. The serum SDF-1α level in each sample was quantitatively analyzed using an SDF-1α ELISA kit; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Immunofluorescent microscopy

The grafted skin pieces were harvested and subjected to immunofluorescent analysis. The excised skin pieces were soaked overnight in 4% paraformaldehyde, embedded in Tissue-Tec OCT Compound (Sakura Finetek, Torrance, CA), frozen on dry ice, and stored at −80°C. For immunofluorescent staining, 7-μm-thick sections were incubated with goat polyclonal anti-mouse Col7 Ab (generated in our laboratory), goat polyclonal anti-mouse PDGFRα Ab (1:200; R&D Systems), rat monoclonal anti-mouse CD31 Ab (1:50; BD Pharmingen, San Diego, CA), rabbit monoclonal anti-mouse cytokeratin 5 (K5) Ab (1:500; Abcam), rat monoclonal anti-mouse neutrophil marker Ab (1:200; Santa Cruz Biotechnology, Dallas, TX), and rat monoclonal anti-mouse CD68 Ab (1:200; Abcam, Cambridge, MA) followed by Alexa Fluor 546-conjugated donkey anti-goat IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 546-conjugated goat anti-rat IgG (1:400; Molecular Probes) as the secondary Abs. The sections were then stained with DAPI and mounted with VECTA Shield anti-fade solution (Vector Laboratories). All images were obtained using a Nikon model A1/C1 confocal laser microscope using NIS-Elements AR 3.1 software (Nikon).

Flow cytometry and cell sorting

The grafted skin was harvested and cut into small pieces using scissors. Tissue pieces were dissociated enzymatically in DMEM (Nacalai Tesque, Kyoto, Japan) containing 0.2% collagenase A (Roche Diagnostics, Tokyo, Japan) at 37°C for 1 h with gentle agitation. The obtained cell suspensions were filtered through a cell strainer. Bone marrow cells were isolated as described above. The fluorescence-conjugated Abs used in this study were as follows: APC anti-mouse lineage mixture with isotype control (BD Pharmingen), PE anti-mouse PDGFRα Ab (eBioscience, San Diego, CA), APC anti-mouse PDGFRα Ab (eBioscience), PE anti-mouse CXCR4 Ab (BD Biosciences), and Alexa Fluor 647-conjugated anti-mouse CXCR4 Ab (BioLegend, San Diego, CA). The stained cells were analyzed using a FACSCanto II device (BD Biosciences) and FlowJo 7.6.1 software (Tree Star, Portland, OR).

For the cell sorting experiments, a BD FACSAria II device (BD Biosciences) was used. Sorting gates were defined based on isotype control staining. RNA from the sorted cells was obtained using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions and then subjected to conventional RT-PCR.

Real-time PCR

The engrafted skin was harvested and subjected to real-time PCR analysis. Total RNA was prepared using an RNeasy RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). mRNA was quantified by real-time PCR using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan). Real-time PCR was performed and analyzed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and the following primer sets: PDGFRα: 5′-GACGAGTGTCCTTCGCCAAAGTG-3′ (forward) and 5′-CAAAATCCGACCAAGCACGAGG-3′ (reverse); CD11b: 5′-CAATAGCCAGCCTCAGTGC-3′ (forward) and 5′-GAGCCCAGGGGAGAAGTG-3′ (reverse); F4/80: 5′-AAGCATCCGAGACACACACA-3′ (forward) and 5′-GGCAAGACATACCAGGGAGA-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-ACTCCCACTCTTCCACCTTC-3′ (forward) and 5′-TCTTGCTCAGTGTCCTTGC-3′ (reverse);

SDF-1α: 5′-CTGTGCCCTTCAGATTGTTG-3′ (forward) and 5′-TAATTTCGGGTCAATGCACA-3′ (reverse); TATA box binding protein (TBP): 5′-ACGGACAACTGCGTTGATTT-3′ (forward) and 5′-TTCTTGCTGCTAGTCTGGATTG-3′ (reverse). Col7a1 was detected using commercially designed primers (Qiagen). The expression level of SDF-1α was normalized to TBP.

Delivery of CXCR4 antagonist

To ensure sufficient levels of the antagonist throughout the experimental period, we used osmotic Alzet (Alza Corporation, Vacaville, CA) pumps to deliver the CXCR4 antagonist AMD3100 (Sigma-Aldrich, St. Louis, MO) at a constant rate of 10 mg/kg/day. The Alzet pumps were loaded with AMD3100 or PBS and implanted s.c. 1 h before skin graft.

Statistical analysis

Statistical analyses were performed using JMP 8 software. The results are presented as the mean ± SEM. Statistical significance was evaluated using unpaired Student t tests for comparisons between two groups or using ANOVAs for multiple comparisons; $p < 0.05$ was considered statistically significant.

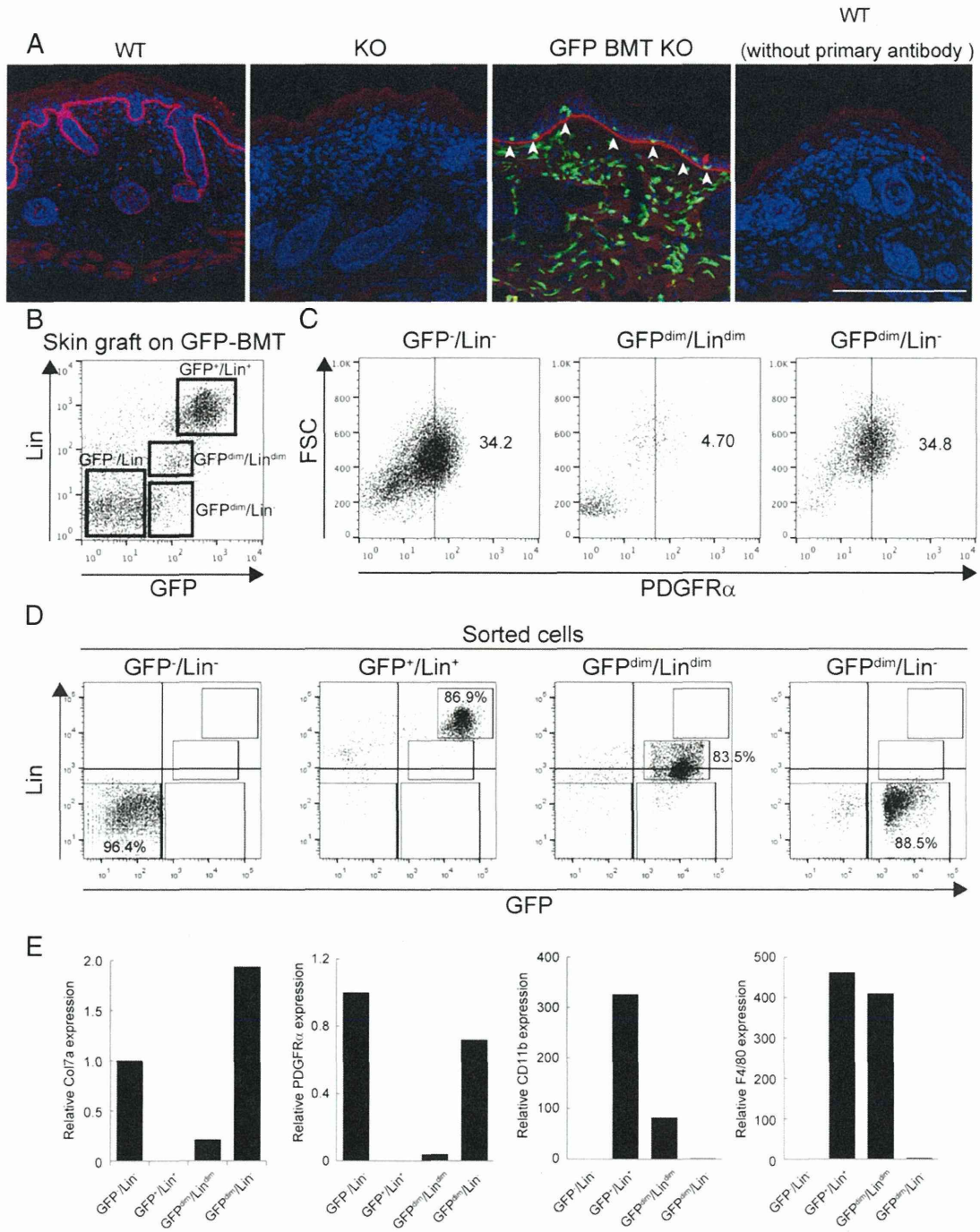

FIGURE 1. Col7 supplementation by bone marrow–derived nonhematopoietic cells. (**A**) Immunostaining of a Col7-null mouse skin graft on a GFP-BMT mouse at day 28. Red, Col7; green, GFP; Blue, DAPI. Scale bar, 50 μm. Arrowheads point to the basement membrane zone. (**B**) Flow cytometric analysis of cells in Col7-null mouse skin grafts on GFP-BMT mice at day 28. (**C**) Flow cytometric analysis of PDGFRα expression in cells from Col7-null skin grafts. (**D**) FACS cell profiles. (**E**) Gene expression of Col7, PDGFRα, CD11b, and F4/80 in sorted cells. GAPDH was used as an internal control.

Results

Transplanted bone marrow–derived non-hematopoietic cells, including PDGFRα$^+$ mesenchymal cells, provide Col7 to RDEB mouse skin grafts

We first examined the particular cell populations that produce Col7 in RDEB mouse skin engrafted onto wild-type mice treated with GFP-BMT after a lethal dose of radiation. Increased numbers of GFP$^+$ bone marrow–derived cells migrated to the RDEB mouse skin grafts, resulting in a restoration of Col7 at the basement membrane zone by day 28 after skin grafting (Fig. 1A). To determine the particular population of bone marrow–derived cells supplying Col7 to the engrafted RDEB mouse skin, we performed flow cytometric analysis of Col7-null skin grafts and identified four populations: GFP$^-$/Lin$^-$ cells, GFP$^+$/Lin$^+$ cells, GFP^{dim}/Lin^{dim} cells, and GFP^{dim}/Lin$^-$ cells (Fig. 1B). In addition, flow cytometry also showed that PDGFRα was predominantly expressed by GFP^{dim}/Lin$^-$ cells, suggesting that this population includes bone marrow–derived MSCs (Fig. 1C). The GFP$^-$/Lin$^-$ cell population, which likely includes resident skin keratinocytes and fibroblasts, also expressed PDGFRα. Then, the four populations of the Col7-null skin grafts were separated using FACS (Fig. 1D), and the gene expression profile of each population (GFP$^-$/Lin$^-$ cells, GFP$^+$/Lin$^+$ cells, GFP^{dim}/Lin^{dim} cells, and GFP^{dim}/Lin$^-$ cells) was analyzed (Fig. 1E). Real-time PCR analysis indicated that GFP^{dim}/Lin$^-$ cells highly expressed Col7a1 in Col7-null skin grafts. However, GFP$^-$/Lin$^-$ cells also expressed Col7a1, suggesting that resident cells from the intact skin adjacent to the skin graft migrated to the Col7-null skin graft and produced Col7a1. The expression levels of Col7a1 and PDGFRα were barely detectable in GFP$^+$/Lin$^+$ cells and GFP^{dim}/Lin^{dim} cells. Instead, GFP$^+$/Lin$^+$ cells and GFP^{dim}/Lin^{dim} cells highly expressed CD11b, a marker for macrophages, NK cells, and granulocytes, as well as F4/80, a marker for pan-macrophages (Fig. 1E). These results suggest that GFP$^+$/Lin$^+$ cells and GFP^{dim}/Lin^{dim} cells include hematopoietic lineage cell populations, such as monocytes and macrophages. Taken together, these data suggest that Col7a1 was primarily supplied by bone marrow–derived nonhematopoietic cells, including the PDGFRα$^+$ MSC population (GFP^{dim}/Lin$^-$). Because mouse MSCs express Col7 in culture (28), we next focused on the mechanism of bone marrow–derived PDGFRα$^+$ cell migration to the grafted skin.

Specific accumulation of bone marrow–derived PDGFRα$^+$ cells in grafted skin

We then examined the accumulation of bone marrow–derived GFP$^+$/PDGFRα$^+$ cells in the wild-type skin grafted on the backs of GFP-BMT mice using immunofluorescent analysis. Because dermal fibroblasts also express PDGFRα, many GFP$^-$/PDGFRα$^+$ cells were observed in the dermis of the graft. Among these cells, GFP$^+$/PDGFRα$^+$ cells were disseminated over the entire dermis of the skin graft, indicating that bone marrow–derived PDGFRα$^+$ cells migrated into the skin graft (Fig. 2A).

To analyze the GFP$^+$/PDGFRα$^+$ cells quantitatively in the skin graft, the day 1, day 4, and day 7 grafts were harvested, and single-cell suspensions of these skin grafts were then subjected to flow cytometric analysis. The quantitative analysis indicated a gradual and significant elevation of the GFP$^+$/PDGFRα$^+$ cell population in the skin graft, reaching over 1.0% of all cells in the day 7 graft (Fig. 2B, 2C). Nongrafted areas in the skin of the same mice did not show such an increase (Fig. 2C), which strongly suggests the existence of a specific recruiting mechanism in the grafted skin. It should be noted, however, that bone marrow PDGFRα$^+$ cells had already significantly migrated into the nongrafted skin of the mice at day 0 (Fig. 2C), albeit at lower levels than in the grafted skin. This migration was possibly the result of a different recruiting mechanism induced by lethal dose irradiation-induced cutaneous injury or another intrinsic mechanism of the skin.

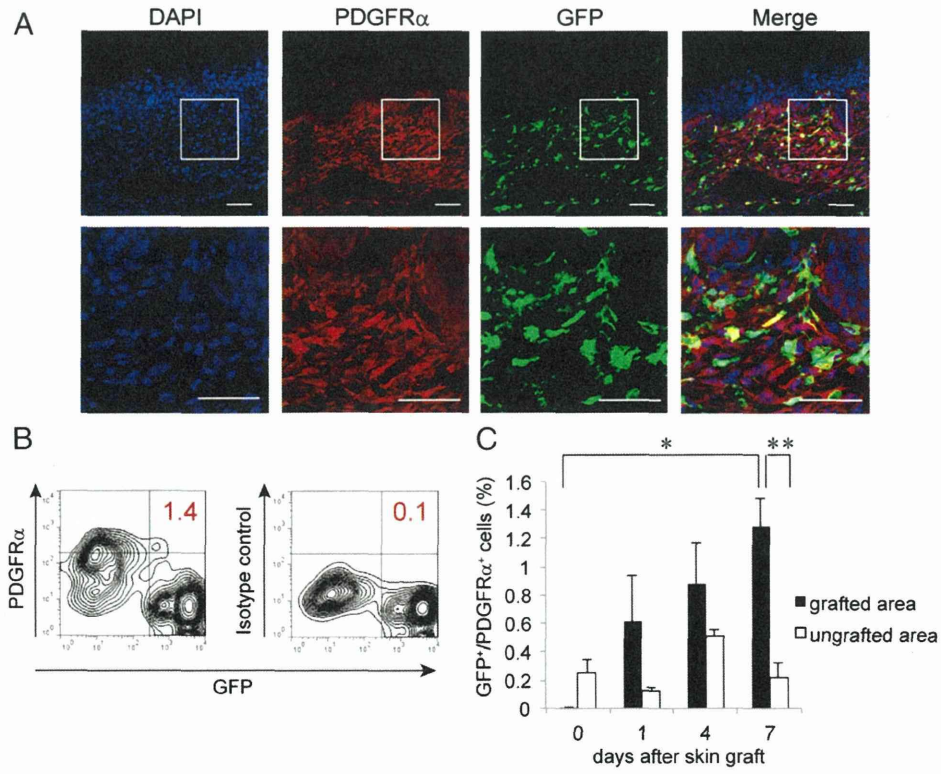

FIGURE 2. Recruitment of bone marrow–derived PDGFRα$^+$ cells into grafted skin. (**A**) Immunostaining of grafted skin on a GFP-BMT mouse at day 7. A portion of bone marrow-derived cells (GFP$^+$) stained positively for PDGFRα in the dermis. Green, GFP; red, PDGFRα; blue, DAPI. Scale bars, 50 μm. The boxed region is displayed in lower panels at a higher magnification. (**B**) Flow cytometric analysis of cells obtained from grafted skin on GFP-BMT mice at day 4. GFP and PDGFRα double-positive cells were detected in grafted skin. (**C**) Time course analysis of GFP$^+$/PDGFRα$^+$ cell migration in grafted skin and nongrafted skin. (n = 4 per group) Values are the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

Endothelial and follicular cells are sources of SDF-1α in grafted skin

To determine whether the SDF-1α/CXCR4 axis plays a specific role in recruiting bone marrow–derived circulating PDGFRα$^+$ cells into the grafted skin, we then compared SDF-1α expression in the grafted and non-grafted areas of the skin. Real-time PCR analysis revealed that SDF-1α expression was significantly increased in the skin graft after transplantation, and the maximal increase was observed in the day 4 graft (Fig. 3A). By contrast, no such increase was observed in the non-grafted area (Fig. 3A). These data suggest a critical role of SDF-1α in the graft-specific recruiting mechanism. However, the serum SDF-1α level did not increase after skin grafting (Fig. 3B), which suggests a regional rather than a systemic role of SDF-1α in recruitment.

To determine the particular cell population in the skin graft releasing SDF-1α, we examined SDF-1α expression in skin grafted from a SDF-1α/GFP knock-in mouse onto wild-type mice

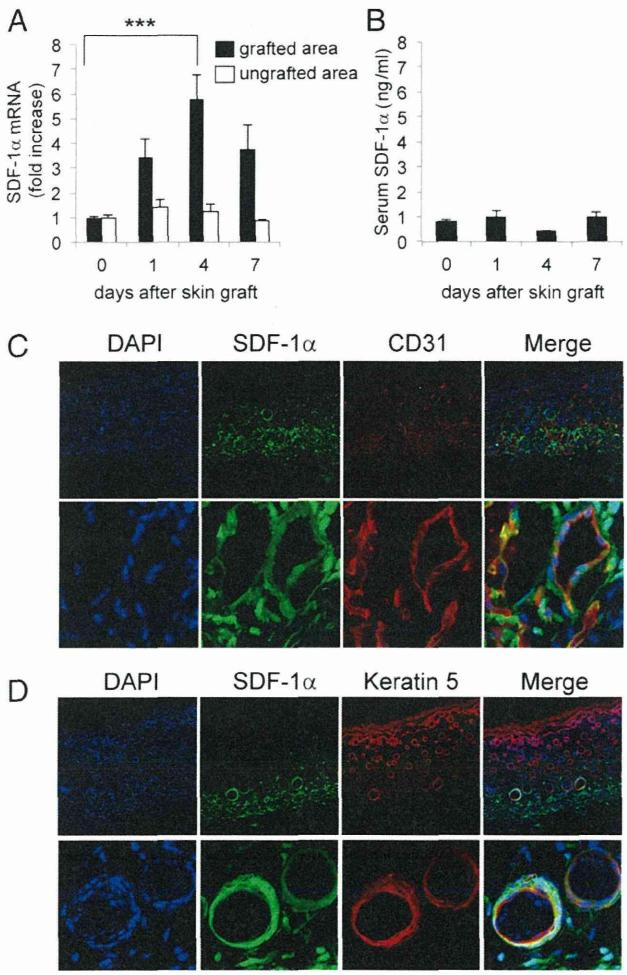

FIGURE 3. SDF-1α expression in grafted skin. (**A**) SDF-1α mRNA expression normalized by TBP (internal control) was determined using real-time PCR at the indicated time points in grafted skin and nongrafted skin. Data are expressed as the fold increase versus the nontreated control (day 0). Values are the mean ± SEM. $n = 4$ per group. ***$p < 0.001$. (**B**) SDF-1α levels in the serum were determined using ELISA at the indicated time points after skin grafting. (**C** and **D**) Immunostaining with CD31 (C) or keratin 5 (K5) (D) of a day 4 skin graft of a SDF-1α/GFP knock-in mouse. SDF-1α was colocalized with both CD31 and K5 in the deep dermis of the grafted skin. Lower panels show the colocalized regions at higher magnification. Green, SDF-1α; red, CD31, K5; blue, DAPI. Scale bars, 100 μm. $n = 4$ per group.

at day 4. The SDF-1α/GFP signal was detected in the deep dermal cells of the graft (Fig. 3C). A previous report indicated that endothelial cells are major sources of SDF-1α in the dermis of hypoxic skin flaps (23). We therefore stained the day 4 graft samples with the endothelial cell marker CD31 and the keratinocyte marker cytokeratin 5 (K5). As expected, CD31$^+$ cells in the deep dermis of the graft were costained with SDF-1α/GFP (Fig. 3C, 3D), which indicates that CD31$^+$ endothelial cells in the deep dermis were the sources of SDF-1α in the grafted skin. It is particularly interesting that follicular keratinocytes expressing K5 in the deep dermis, and not epidermal cells, also showed significant SDF-1α expression. Because cultured epidermal keratinocytes and separated epidermal sheets from the skin grafts did not show SDF-1α expression via real-time PCR (data not shown), a follicular keratinocyte-specific recruiting mechanism for CXCR4$^+$ cells is suggested.

The SDF-1α/CXCR4 axis is essential for recruiting bone marrow–derived PDGFRα$^+$ cells to grafted skin

To confirm that the SDF-1α/CXCR4 axis plays an essential role in the specific recruitment of bone marrow–derived PDGFRα$^+$ cells to the grafted skin, we then analyzed the expression of CXCR4 on PDGFRα$^+$ cells in both the bone marrow and the day 4 grafts. In freshly isolated mouse bone marrow cells, expression of CXCR4 was detected on PDGFRα$^+$ cells via flow cytometry (Fig. 4A). CXCR4 expression was also observed on the surface of GFP$^+$/PDGFRα$^+$ cells from day 4 grafts on GFP-BMT mice (Fig. 4B). To assess the role of CXCR4 in recruiting PDGFRα$^+$ cells to the skin grafts, we systemically administered the CXCR4 antagonist AMD3100 using an osmotic pump implanted s.c. into GFP-BMT mice prior to skin grafting. A drastic reduction of GFP$^+$/PDGFRα$^+$ cell migration was observed in skin grafts on the mice systemically administered with AMD3100, but not with PBS (Fig. 4C, 4D). These data demonstrate a critical role of the SDF-1α/CXCR4 axis in the specific recruitment of PDGFRα$^+$ bone marrow cells to grafted skin.

PDGFRα$^+$ cells play a pivotal role in the regeneration of RDEB mouse skin grafts

We examined the effects of blocking PDGFRα$^+$ cell migration on the regeneration of RDEB mouse skin grafts. Without AMD3100 administration, linear deposition of Col7 along the dermal–epidermal junction was clearly restored throughout day 14 graft of RDEB mouse skin (Fig. 5A). As a result, a regenerated epidermis was maintained without significant blistering in the day 14 graft (Fig. 5B). By contrast, with systemic AMD3100 administration, Col7 restoration was almost completely interrupted at the dermal–epidermal junction of the Col7-null mouse skin graft (Fig. 5A), resulting in separation and degeneration of the epidermis with massive inflammatory cell infiltration in the dermis (Fig. 5B). These data suggest that the SDF-1α/CXCR4 axis-mediated migration of bone marrow–derived PDGFRα$^+$ cells is essential for restoring Col7 in the cutaneous basement membrane zone of Col7-null mouse skin grafts.

For further analysis of the increased infiltration of mononuclear cells in AMD3100-treated mice, we performed immunostaining with a neutrophil marker and CD68, which is a cell surface marker for macrophages. There was an increase in neutrophil marker-positive cells in the AMD3100-treated group (Fig. 5C). Furthermore, there was a significant increase in the number of CD68 positive cells in the AMD3100-treated group (Fig. 5D). These data suggest that CXCR4-antagonist treatment of Col7-null skin grafted mice also enhanced inflammation in the skin grafts by increasing the infiltration of neutrophils and macrophages.

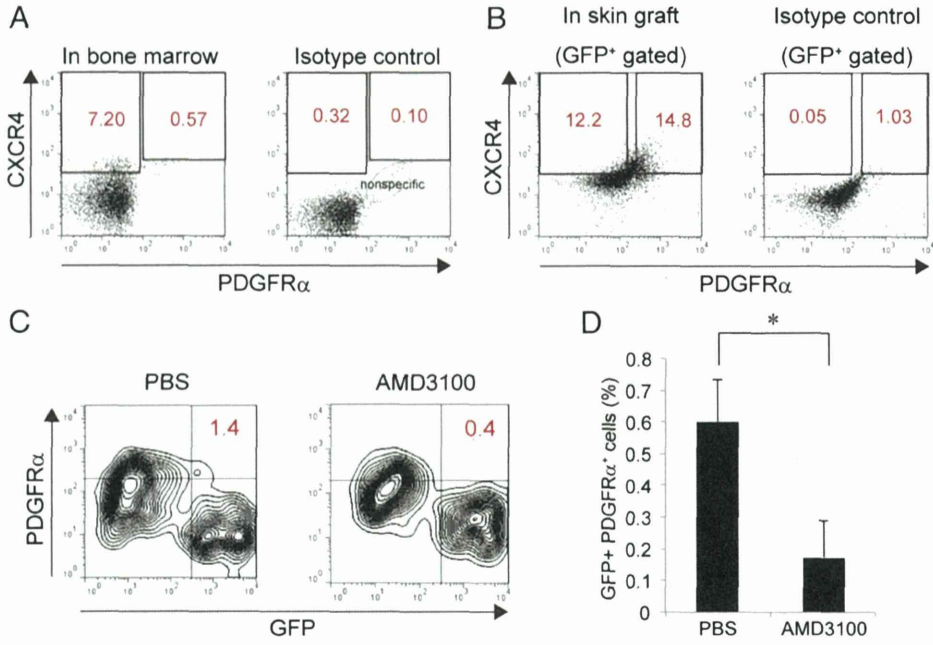

FIGURE 4. Effect of a CXCR4 antagonist on PDGFRα+ cell migration into the grafted skin. (**A**) Flow cytometric analysis of CXCR4 in freshly isolated PDGFRα+ bone marrow cells. Some PDGFRα+ cells in the bone marrow expressed CXCR4. (**B**) Flow cytometric analysis of CXCR4 on GFP+/PDGFRα+ cells that had migrated into skin grafted onto GFP-BMT mice by day 4. CXCR4+/GFP+/PDGFRα+ cells were observed in the grafted skin. (**C**) Flow cytometric analysis of GFP+/PDGFRα+ cells in skin grafted onto GFP-BMT mice at day 4 with or without treatment of the CXCR4 antagonist AMD3100. (**D**) Quantitative analysis of GFP+/PDGFRα+ cells in grafted skin using flow cytometry on day 4 with or without AMD3100 treatment. The migration of GFP+/PDGFRα+ cells into grafted skin was significantly blocked by AMD3100. $n = 4$ per group. Values are the mean ± SEM. $*p < 0.05$.

Discussion

In this study, we provide evidence that transplanted bone marrow-derived nonhematopoietic cells, including PDGFRα+ cells, play a crucial role in regenerating the skin of RDEB mice by restoring Col7 in the cutaneous basement membrane zone following BMT. We also demonstrate an indispensable role of the SDF-1α/CXCR4 axis for recruiting bone marrow–derived PDGFRα+ cells to grafted skin, in which blood circulation is initially terminated, resulting in a severely hypoxic/necrotic condition. These data support the notion of allogeneic BMT as a novel therapeutic option for severely affected RDEB patients who have impaired Col7 expression and numerous necrotic lesions in the skin.

PDGFRα is an established marker for mouse bone marrow MSCs, which include ectodermally derived multipotent stem cells (29). Intravenously transplanted cultured mouse MSCs were previously shown to accumulate in wounded skin and differentiate into multiple skin cell types, including fibroblasts, endothelial cells, pericytes, and keratinocytes (30). Bone marrow–derived PDGFRα+ cells can also differentiate into ectodermal keratinocytes and mesenchymal dermal fibroblasts, particularly in the setting of skin grafts (14). Bone marrow–derived keratinocytes were observed in skin grafts up to 5 mo after transplantation in a previous report, suggesting supplementation of resident epidermal progenitor/stem cells from the bone marrow. In this study, we detected bone marrow–derived mesenchymal cells in the dermis in day 7 skin grafts, suggesting that bone marrow–derived PDGFRα+ cells primarily serve as mesenchymal cells, such as fibroblasts, in the dermis of a skin graft or wound, but have the potential to become keratinocytes in a particular milieu or niche to induce an epigenetic transition from mesenchymal to epithelial lineages, particularly in RDEB skin (14).

In addition to their multidifferentiation potential, MSCs have been shown to promote wound healing processes by providing various trophic factors in lesions. For example, MSCs locally administered into injured tissue promote neovascularization by releasing proangiogenic cytokines, such as vascular endothelial growth factor-α, insulin-like growth factor-1, PDGF-BB, and angiopoietin-1 (31, 32). Recently, transplanted MSCs were shown to suppress immune and inflammatory reactions by releasing anti-inflammatory molecules, including IL-10, PG-E, and TNF-stimulated gene-6 protein (33–36). In the current study, the massive inflammatory reaction observed in the skin grafts when PDGFRα+ cell migration was blocked suggests that these cells also have anti-inflammatory activity. Therefore, the migration of bone marrow–derived PDGFRα+ cells seems to play multiple roles in the regeneration of injured skin and in the engraftment of skin grafts.

Accumulating evidence, including that presented in this study, has defined a crucial role of the SDF-1α/CXCR4 axis in recruiting bone marrow–derived MSCs for the regeneration of tissue in conditions such as bone fractures (21), brain damage (37), and infarcted myocardium (38). However, several reports have provided contradictory results. One report found that MSCs lack many effectors of homing, particularly CXCR4 (39), whereas another study indicated that MSCs use β1 integrin, not CXCR4, for myocardial migration and engraftment (40). This discrepancy may be partially explained by the amount or duration of SDF-1α expression in damaged tissue. In this context, augmentation of the SDF-1α/CXCR4 axis by overexpression, drug treatment, or both may enhance further recruitment of MSCs to various types of tissue damage. This concept could be a promising therapeutic strategy for the effective delivery of MSCs.

We demonstrate in this study that transplanted bone marrow–derived PDGFRα+ mesenchymal cells migrated to donor RDEB mouse skin and supplemented Col7 in the basement membrane zone. Our previous work showed that embryonic transfer of bone marrow cells into the circulation of RDEB mice resulted in restoration of Col7 via the engraftment of bone marrow–derived

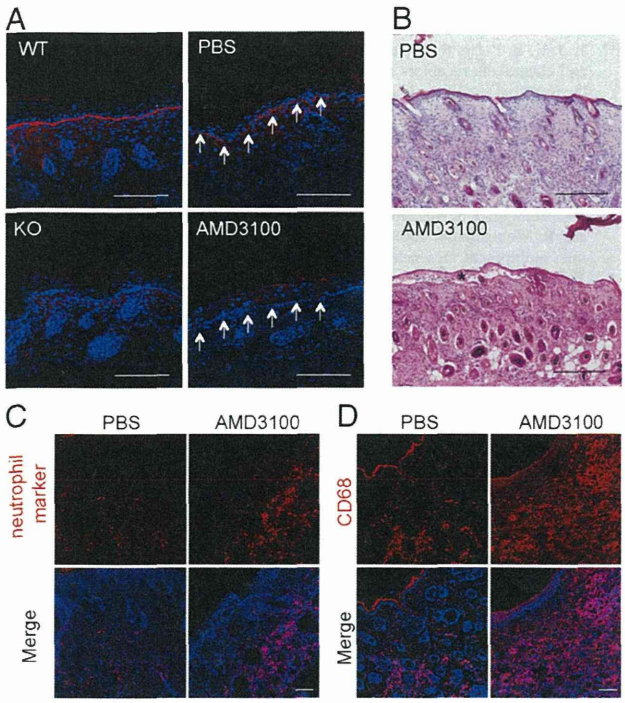

FIGURE 5. Effect of a CXCR4 antagonist on grafted Col7-null mouse skin. (**A**) Immunostaining of Col7-null skin grafted on a wild-type mouse at day 14. Arrows point to the basement membrane region of the dermal–epidermal junction. (**B**) Hematoxylin-eosin staining of grafted Col7-null skin on a wild-type mouse at day 14. Asterisk indicates the blister cavity. (**C**) Immunostaining for neutrophil markers and (**D**) CD68 in Col7-null skin grafted on a wild-type mouse at day 14 with or without AMD3100 treatment. KO, Col7 knockout mouse skin graft (negative control); PBS, PBS-administered Col7 knock-out mouse skin graft, AMD3100, AMD3100-administered Col7 knockout mouse skin graft; WT, wild-type mouse skin graft (positive control). Red, Col7; blue, DAPI. Scale bars, 100 μm.

fibroblasts (1). Col7 is believed to be produced predominantly by epidermal keratinocytes and less so, but still at physiologically relevant levels, by dermal fibroblasts (1, 41–43). Our study suggests that bone marrow MSCs may supplement Col7 in a third-party manner by differentiating into circulating PDGFRα$^+$ mesenchymal cells, which then migrate to the injured skin and differentiate into not only dermal fibroblasts but also epidermal keratinocytes if the epidermis is severely damaged (14). In this context, it is noteworthy that transplanted cultured MSCs were previously shown to supply Col7 to the dermal–epidermal junction of Col7-null mice and Col7-null RDEB patients, thereby preventing blistering (28, 44).

Collectively, our data together with previous reports suggest that bone marrow–derived mesenchymal cells, including PDGFRα$^+$ cells, could be a putative source of Col7 in RDEB patient skin, although the role of PDGFRα$^+$ bone marrow cells in the human setting needs to be investigated further for future clinical applications.

Disclosures

The authors have no financial conflicts of interest.

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