Stem Cells, Tissue Engineering and Hematopoietic Elements

Bone Marrow Cell Transfer into Fetal Circulation Can Ameliorate Genetic Skin Diseases by Providing Fibroblasts to the Skin and Inducing Immune Tolerance

Takenao Chino,*[†] Katsuto Tamai,*
Takehiko Yamazaki,* Satoru Otsuru,*
Yasushi Kikuchi,* Keisuke Nimura,*
Masayuki Endo,* Miki Nagai,[†] Jouni Uitto,[‡]
Yasuo Kitajima,[†] and Yasufumi Kaneda*

From the Division of Gene Therapy Science,* Osaka University Graduate School of Medicine, Osaka, Japan; the Department of Dermatology,[†] Gifu University School of Medicine, Gifu, Japan; and the Department of Dermatology and Cutaneous Biology,[‡] Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania

Recent studies have shown that skin injury recruits bone marrow-derived fibroblasts (BMDFs) to the site of injury to accelerate tissue repair. However, whether uninjured skin can recruit BMDFs to maintain skin homeostasis remains uncertain. Here, we investigated the appearance of BMDFs in normal mouse skin after embryonic bone marrow cell transplantation (E-BMT) with green fluorescent protein-transgenic bone marrow cells (GFP-BMCs) via the vitelline vein, which traverses the uterine wall and is connected to the fetal circulation. At 12 weeks of age, mice treated with E-BMT were observed to have successful engraftment of GFP-BMCs in hematopoietic tissues accompanied by induction of immune tolerance against GFP. We then investigated BMDFs in the skin of the same mice without prior injury and found that a significant number of BMDFs, which generate matrix proteins both *in vitro* and *in vivo*, were recruited and maintained after birth. Next, we performed E-BMT in a dystrophic epidermolysis bullosa mouse model (col7a1^{-/-}) lacking type VII collagen in the cutaneous basement membrane zone. E-BMT significantly ameliorated the severity of the dystrophic epidermolysis bullosa phenotype in neonatal mice. Type VII collagen was deposited primarily in the follicular basement membrane zone in the vicinity of the BMDFs. Thus, gene therapy using E-BMT into the fetal circulation may offer a potential treatment option to

ameliorate genetic skin diseases that are characterized by fibroblast dysfunction through the introduction of immune-tolerated BMDFs. (*Am J Pathol* 2008, 173:803–814; DOI: 10.2353/ajpath.2008.070977)

Bone marrow (BM) has been shown to harbor progenitor cells of bone marrow-derived fibroblasts (BMDFs) that have the propensity to migrate into injured tissues, including the skin.^{1–7} Previous studies have shown that wounded skin recruits BMDFs that become resident cells and accelerate tissue repair and wound-healing processes.^{8,9} However, whether BMDFs can contribute to healthy skin without prior injury to maintain homeostasis of the structure and function remains unclear, in part because marker gene-transgenic bone marrow transplantation (BMT) for tracing the fate of the transplanted cells in mice requires lethal doses of irradiation that injures all tissues including the skin. Consequently, we have now investigated the fate of the transplanted bone marrow cells (BMCs) in the skin under physiological conditions without irradiation and injury.

Embryonic transplantation of congenic, and possibly allogenic, BMCs transduced with a marker gene, such as green fluorescent protein (GFP), in animal models showed that the transplanted cells successfully engrafted in the recipient BM for long periods without prior

Supported by health science research grants for research on specific disease from the Ministry of Health, Labor, and Welfare of Japan; by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and by the National Institutes of Health, U.S.A.

Accepted for publication May 29, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Katsuto Tamai, M.D., Ph.D., or Yasufumi Kaneda, M.D., Ph.D., Division of Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: tamai@gts.med.osaka-u.ac.jp, and kaneday@gts.med.osaka-u.ac.jp.

myeloablative regimen, such as lethal irradiation, and generated hematopoietic chimerism by inducing immune tolerance.¹⁰ In this context, embryonic-BMT (E-BMT) might allow us to evaluate the potency of BM to raise BMDFs in normal skin without injury. However, previous E-BMT mouse protocols are rather invasive to the embryo with a high incidence of embryonic death. Furthermore, there is a limitation to the number of cells for transplantation because such E-BMT procedures require direct injection of the BMCs into the fetus by intraperitoneal, subcutaneous, or intrahepatic approaches.¹¹

Recently, a far less invasive E-BMT via the vitelline vein, which is located under the uterine wall and directly communicates with the embryonic circulation, was shown to generate efficient hematopoietic chimerism.¹² Because this E-BMT procedure allows the transfer of as many as 1.0×10^6 cells/embryo without embryonic tissue damage, we can trace the fate of the transplanted GFP-transgenic bone marrow cells (GFP-BMCs) in the hematopoietic and nonhematopoietic tissues of the mice undergoing physiological development during the fetal and postnatal periods.

Clinically, E-BMT via fetal circulation has been applied to human hematopoietic and enzyme storage diseases, such as severe combined immunodeficiency (SCID) and leukodystrophy, to provide functional hematopoietic lineage of cells in conjunction with inducing immune tolerance against the transplanted allogenic cells.¹³ For non-hematopoietic tissue diseases, however, the therapeutic potential of E-BMT has not been well established. If E-BMT can generate a significant number of immunologically tolerated allogenic BMDFs that synthesize matrix molecules, such as collagen, in the skin after birth, it may provide a therapeutic option for inherited skin diseases with defective matrix molecules attributable to genetic fibroblast dysfunction.

Dystrophic epidermolysis bullosa (DEB) is a family of inherited mechanobullous skin disorders caused by mutations in the COL7A1 gene that encodes type VII collagen necessary for stable epidermal-dermal adherence.^{14–16} Previous studies have suggested that both epidermal keratinocytes and dermal fibroblasts are capable of synthesizing type VII collagen.^{17–19} It was then demonstrated that transplanted dermal fibroblasts were capable of producing type VII collagen at the cutaneous basement membrane zone.^{20,21} Another study showed that type VII collagen-transgenic fibroblasts supplied type VII collagen to the dermal-epidermal junction more efficiently than gene-transgenic keratinocytes.²² More recently, Wong and colleagues²³ demonstrated the clinical potential of allogenic fibroblast cell therapy for recessive DEB (RDEB) patients. These studies suggest that transplantation of functional fibroblasts may be a promising therapeutic option for DEB treatment.²⁴

In case of the most severe, so-called Hallopeau-Siemens type of RDEB (HS-RDEB), the patients frequently harbor nonsense mutations in both alleles of COL7A1 and demonstrate no expression of the corresponding gene, leading to the suggestion that HS-RDEB patients might not have immune tolerance against type VII collagen molecules if introduced by gene therapy. If this is

indeed the case, therapies with allogenic fibroblasts or recombinant type VII collagen may result in failure because of antibody formation and immunological rejection. To overcome such difficulties, E-BMT may be an ideal procedure for this disease, not only by providing functional BMDFs but also inducing immune tolerance against allogenic BMDFs expressing type VII collagen.

In this study, we searched for evidence of BMDF generation in normal mouse skin after E-BMT with congenic GFP-BMCs via the vitelline vein. We also evaluated immune tolerance induction against an exogenously introduced non-self molecule, GFP, by E-BMT with the GFP-BMCs. Finally, we investigated the therapeutic potential of E-BMT to DEB model mice, which ordinarily die within a few days after birth because of skin separation. We succeeded in showing, for the first time, that E-BMT could be a therapeutic option for DEB by providing type VII collagen molecules from functional BMDFs.

Materials and Methods

Mouse Recipients and Donors

Day 12 to 13 embryos of C57BL/6 mice were BMT recipients, and GFP-transgenic C57BL/6 donor mice were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan).

Preparation of Donor BMCs

Adult GFP⁺ BMCs (GFP-BMCs) were isolated from GFP-transgenic mice (C57BL/6 background) 8 weeks after birth by flushing the tibiae and femurs with RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum using a 27-gauge needle. After filtration through a 40- μ m nylon mesh filter, GFP-BMCs were centrifuged at $440 \times g$ for 7 minutes at 24°C. CD90⁺ T cells were magnetically depleted by negative selection with anti-CD90 (Miltenyi Biotec, Gladbach, Germany). The GFP-BMCs were counted and suspended in Ca/Mg-free phosphate-buffered saline (PBS; Nacalai Tesque) at a density of 1.0×10^8 cells/ml for injection.

Embryonic BMC Transplantation via the Vitelline Vein

Embryonic BMC transplantation via the vitelline vein was performed as described previously.¹⁰ Briefly, pregnant mice (C57BL/6) on days 12 to 13 of gestation were anesthetized, and the uterus was exposed through a midline laparotomy incision under sterile conditions. Beveled glass micropipettes were placed under stereoscopic microscopy into the vitelline vein (Figure 1A), which directly communicates with the fetus *in utero*. Each injection contained 1.0×10^6 viable GFP-BMCs in 10 to 20 μ l of PBS. In the case of the DEB model mice, each injection contained 0.5×10^6 viable GFP-BMCs in 10 to 20 μ l of PBS. For an unknown reason, all embryos of the DEB model mice died when more than 0.5×10^6 viable GFP-BMCs was injected. The uterus and fetus were returned to the abdomen, which was closed using a 4–0 silk suture. For

Figure 1. Successful delivery of BMCs to mouse embryos via the vitelline vein. **A:** Vitelline vein (arrow) of a day E13 embryo in amnion under stereoscopic microscope observation. **B:** Successful injection of toluidine blue-containing solution via the vitelline vein at day E13 (right, white arrow, left, an uninjected control). **C:** Day E13 mouse skin at 30 minutes after GFP-transgenic E-BMT via the vitelline vein. Original magnification, $\times 80$.

each pregnancy, GFP-BMCs were injected on the average into seven fetuses via the vitelline vein within 1 hour. The uterus was frequently flooded with warm sterile saline during the procedure to prevent drying and to maintain the maternal temperature.

Fluorescence Stereoscopic Microscopy

All of the mouse organs at 11 to 12 weeks after the embryonic GFP-BMT were directly observed under a microscope equipped with a mercury lamp (Leica Microsystems AG, Wetzlar, Germany) and GFP filters.

Analysis of Bone Marrow Chimerism

Recipients of GFP-BMT were euthanized between 2 to 6 months after embryonic BMT and the BMCs were harvested by flushing the tibiae and femurs with PBS using a 28-gauge needle. After passage through a 40- μ m nylon mesh filter, the red blood cells were lysed for 1 to 2 minutes using ACK lysing buffer (Cambrex Bio Science, Walkersville, MD). Samples were analyzed using a FACScan (Becton Dickinson, San Diego, CA).

Skin Grafting

Full thickness tail skin from transgenic GFP mice isolated by excision under anesthesia was cut into $\sim 10 \times 10$ -mm squares. The GFP-positive tail skin was engrafted onto the back of recipient mice at 6 weeks after birth just above the muscular fascia and secured with a bandage for 7 days.

Measurement of Antibody Generation against GFP

Tail skin of GFP mice was engrafted onto the back of 6-week-old mice after E-BMT or neonatal subcutaneous transplantation with 1.0×10^6 of GFP-BMCs, GFP fibro-

blasts, or GFP mesenchymal stem cells above the muscular fascia, and secured with a bandage for 7 days. At 4 weeks after GFP skin engraftment, blood samples were collected from tail snips, pooled, and stored at -20°C . Recombinant GFP was diluted to a working concentration of 0.2 $\mu\text{g}/\text{ml}$ in PBS, and 96-well microtiter plates were coated with 100 μl per well and incubated at 4°C overnight. The plates were rinsed twice with washing buffer (0.05% Tween 20 in PBS), and 200 μl of blocking buffer (2% skim milk in PBS) was then added to each well, and the plates were incubated for 2 hours. The antibody harvested from the mice engrafted with GFP skin was diluted 1:250 in PBS, and 100 μl per well was added for 1 hour at room temperature. After rinsing with washing buffer, 100 μl of secondary antibody (anti-mouse IgG, horseradish peroxidase) was added to each well for 1 hour. The microplates were washed and the levels of antibody generated against GFP were measured.

Cytotoxic T-Cell (CTL) Assay against GFP

Mice were anesthetized and splenocytes harvested from isolated spleens by passage through a sterile strainer. The splenocytes were then sedimented by centrifugation at $450 \times g$ for 10 minutes and red blood cells were depleted using ACK buffer for 1 to 2 minutes. The GFP-BMCs from GFP-transgenic mice 8 weeks after birth were harvested, sedimented by centrifugation at $440 \times g$ for 7 minutes at room temperature, resuspended in RPMI 1640, and incubated with Mitomycin C (Nacalai Tesque) for 45 minutes. Finally, isolated splenocytes ($5 \times 10^7/\text{ml}$) were co-cultured with GFP-BMCs ($5 \times 10^6/\text{ml}$) in 75-cm² BD Falcon dishes (BD Biosciences, San Jose, CA) with rhIL-2 at 37°C . After sensitization with the simulator GFP-BMCs for 7 days, the effector splenocytes were harvested and portioned into 96-well tissue culture plates. Responder GFP-BMCs from GFP-transgenic mice were cultured with ^{51}Cr (Amersham BioSciences UK, Ltd., Buckinghamshire, UK) for 30 minutes and mixed with the effector splenocytes for 4 hours. The release of ^{51}Cr from GFP-BMCs disrupted by splenocyte CTL activity into the supernatant was determined by scintillation counting.

Immunofluorescence Analysis

Embryonic BMT mice were fixed by perfusion with 4% paraformaldehyde under anesthesia. The skin tissues were soaked overnight in 4% paraformaldehyde and embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan). The tissues were frozen in liquid nitrogen and stored at -20°C . Sections (6 μm) were cut on a cryostat (Leica Microsystems AG) and fixed in 4% paraformaldehyde for 10 minutes at room temperature. The sections were reacted with rabbit polyclonal anti-mouse type I collagen (1:100), anti-mouse fibronectin (1:100), anti-mouse vimentin (1:100), and anti-mouse type VII collagen (1:200) antibodies, followed by the secondary antibody, Alexa Fluor 546 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR). The sections were finally stained with 4,6-diamidino-2-phenylindole di-

Figure 2. Hematopoietic tissues containing GFP⁺ cells after embryonic BMT. **A:** GFP⁺ cells from GFP⁺ BM are located in BM, spleen, thymus, Peyer's patches, and lymph nodes at 13 weeks after embryonic GFP-BMT. Original magnifications: $\times 40$ (**A, middle**); $\times 80$ (**A, right**). **B:** FACS analysis of BMCs harvested at 12 weeks after embryonic BMT. GFP⁺ cells in BM account for 0.63% of all cells, and more than 99.9% of GFP⁺ BMCs co-express CD45. **C:** Chimerism of GFP⁺ BMCs in each transplantation. The relative amount (%) of donor cells in BM measured by FACSscan is shown. Each point represents the degree of engraftment in BM in embryonic ($n = 5$) and adult ($n = 5$) GFP-BMT mice. The percentage of GFP⁺ cells varies between 0.1% and 0.7% of all cells according to FACS analysis of BMCs from adult mice with embryonic BMT (**left column**). No engraftment of GFP⁺ cells is evident in nonirradiated 8-week-old adult mice with GFP-BMT (**right column**).

hydrochloride, mounted with the antifade solution, Vector Shield (Vector Laboratories, Inc., Burlingame, CA) and covered with a coverslip.

Isolation of Bone Marrow-Derived GFP⁺ Cells from the Dermis of E-BMT Mice

The BM-derived GFP⁺ cells were isolated from the dermis of E-BMT mice at more than 12 weeks of age. Full thickness skin $\sim 10 \times 10$ mm, was isolated from BMT mice isolated by excision under systemic anesthesia, and treated with 1000 PU/ml dispase for 30 minutes at 37°C to separate the epidermis from the dermis. The dermal sheets were then cut into ~ 1 mm squares, incubated for 45 minutes in medium containing bacterial collagenase (Yakult Pharmaceutical Inc., Tokyo, Japan) at 37°C until the skin was visibly dissociated. The cell suspension was grown in Minimal Essential Medium supplemented with 10% fetal bovine serum for 2 to 3 days. The cells were then harvested by trypsinization for 10 minutes and sorted into GFP⁺ cells and GFP⁻ cells with FACSscan (Becton Dickinson) using CellQuest software.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from GFP⁺ BMCs, GFP⁺ fibroblasts, and sorted BM-derived GFP⁺ cells using a RNeasy mini kit (Qiagen, Valencia, CA); 5 μ g of total RNA was reverse-transcribed into the first strand cDNA in a reaction primed by random hexamers primer using Superscript 3 reverse transcriptase (Invitrogen Corp., Carlsbad, CA). First strand cDNA was used as template for PCR reactions using Taq polymerase (Takara Pharmaceutical Inc., Otsu, Japan).

Primers used were as follows: fibronectin: 5'-GAGACAGCCGTGACCCAGACTTA-3' (forward); 5'-CTTCTT-TCCAGCGACCCGTAGAG-3' (reverse), 30 cycles, product size 940 bp; collagen-1: 5'-CTACTCAGCCGTCTGTGC-CT-3' (forward); 5'-GGCAGGGCCAATGTCTAGT-3' (reverse), 30 cycles, product size 450 bp; procollagen 1 α -1: 5'-CCCAGTGGCGGTTATGACTT-3' (forward); 5'-TGAG-GCAGACGGCTGAGTA-3' (reverse), 30 cycles, product size 353 bp; DDR2: 5'-AACCCGATGACCTGAAGGAA-3' (forward); 5'-CTGGGATAAGGCGAACAAAT-3' (reverse),

30 cycles, product size 270 bp; type 7 collagen: 5'-CTCT-TGGCCCCGAGGAAGAG-3' (forward); 5'-GTCCTCGG-GGACCTTCTT G-3' (reverse), 30 cycles, product size 320 bp; GFP: 5'-CTACAAGACCCGCGCCGAGGTGAAG-3' (for-

ward); 5'-GTGACCGCCGCGGGATCACTC-3' (reverse), 30 cycles, product size 376 bp; GAPDH: 5'-TTGAAGGTAGTT-TCGTGGAT-3' (forward); 5'-GAAAATCTGGCACCACAC-CTT-3' (reverse), 30 cycles, product size 265 bp. Reactions were cycled at 95°C for 30 seconds [58°C (fibronectin), 55°C (collagen 1, procollagen 1 α -1, type 7 collagen, GFP, GAPDH), 52°C (DDR2)] for 30 seconds, and 72°C for 1 minute. mRNA transcript-specific amplification products were separated electrophoretically on a 1% agarose gel using All-Purpose Hi-Lo DNA marker (Bionexus Inc., Oakland, CA) for the determination of the amplicon size.

Electron Microscopic Analysis

Skin specimens were fixed with 2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4, for 5 to 7 minutes at 37°C, followed by fixation with a 2% aqueous solution of osmium tetroxide (OsO₄) for 4 hours at 37°C. Fixed samples were embedded in Epon 812 (Nissin EM Company, Ltd., Tokyo, Japan). Ultrathin sections were made parallel to the bottom of the dish and counterstained with uranyl acetate and lead citrate. From some specimens, 4 to 5 serial thin sections (100 nm thick) were made and subjected to standard transmission electron microscopic examination.

Results

E-BMT via the Vitelline Vein Efficiently Distributes Donor Cells to the Fetus

To ensure the effective transfer of transplanted BMCs into fetal circulation, GFP-BMCs were introduced via the vitelline vein, which directly communicates with the fetal circulation, at embryonic day 13 (Figure 1A). In some

Figure 3. Engraftment of GFP-transgenic skin on embryonic GFP-BMT mice and evaluation of humoral and cellular immunity against GFP. **A** and **B**: Skin from GFP-transgenic mouse tail is engrafted 7 weeks after embryonic GFP-BMT. Engrafted GFP transgenic skin under normal (**A**, black arrow) and GFP fluorescent (**B**, white arrow) light. The engrafted GFP-transgenic skin persisted for more than 50 weeks. **C**: Skin graft survival. Survival of GFP skin graft at 6 weeks in embryonic BMT ($n = 5$) and naïve ($n = 5$) mice. Engrafted skin persisted beyond 8 weeks after grafting in all mice that had undergone embryonic BMT. All skin grafts were rejected by nonirradiated adult naïve mice before 5 weeks after grafting. **D**: Generation of antibodies against GFP. Antibodies against GFP in transplanted skin were measured using ELISA. Naïve mice ($n = 4$) produced significant amounts of antibodies (mean $\pm$ SE), whereas embryonic GFP-BMT mice ($n = 6$) did not. **E**: Cytotoxic T-cell (CTL) assay against GFP measured in embryonic GFP-BMT mice ($n = 5$) and GFP-immunized mice ($n = 3$) in response to GFP⁺ BMCs. Assays were performed in triplicate (mean $\pm$ SE). Embryonic mice with GFP-BMT generated no response whereas those with GFP generated significant responses. **F**: Generation of antibodies against GFP. Antibody titers against GFP in the mouse serum after transplantation of GFP-transgenic skin were measured using ELISA. Subcutaneous transplantation of GFP-BMCs (N-scBMT, $n = 4$), GFP-fibroblasts (N-scFT, $n = 7$), and GFP-mesenchymal stem cells (N-scMST, $n = 5$) in neonatal mice did not prevent these mice from generating anti-GFP antibodies after GFP skin transplantation, whereas embryonic GFP-BMT mice (E-BMT, $n = 6$) did not develop anti-GFP antibodies.

Figure 4. Generation of BMDFs by E-BMT in normal skin. **A:** Confocal microscopic picture of adult mouse skin at 13 weeks after GFP-transgenic E-BMT (12 weeks after birth). Note that a significant number of GFP-transgenic BMCs provided by the E-BMT appear scattered on the skin. **B:** Transplanted GFP⁺ BM-derived cells in the adult mouse skin with E-BMT. The majority of the GFP⁺ BM-derived cells resided within the dermal matrix. **C–E:** GFP⁺ BM-derived cells (white arrowheads) were stained with fibroblast marker proteins, including type I collagen (C), fibronectin (D), and vimentin (E). **E:** Asterisks indicate autofluorescence of the hair shafts. **F and G:** Most of GFP⁺ BM-derived cells (white arrowhead) were stained with fibroblastic marker proteins including vimentin around hair follicles. Original magnifications: $\times 40$ (A); $\times 100$ (B); $\times 400$ (C–E); $\times 200$ (F, G).

Figure 5. Characterization of BMDFs in culture. **A:** Immunostaining of the cultured fibroblasts from the adult mouse skin with E-BMT for fibroblastic marker proteins. Note that GFP⁺ BMDFs express fibroblast marker proteins including type I collagen, fibronectin, vimentin, and type VII collagen. **B:** RT-PCR analysis of the sorted BMDFs from the cultured fibroblasts derived from the skin of adult mice with E-BMT. Both the GFP⁺ BMDFs and the fibroblasts from GFP-transgenic mouse skin expressed fibroblast marker genes including fibronectin, type I collagen, procollagen I- α , DDR2, and type VII collagen as well as GAPDH. Note that BMCs of the GFP-transgenic mouse did not express any of those genes except GAPDH. **C:** FACS analysis for percentage of BMDFs in cultured dermal fibroblasts derived from wild-type mice (12 weeks old) with E-BMT (E-BMT, $n = 6$), and immunohistological evaluation for percentage of GFP-positive/vimentin-positive cells in total vimentin-positive cells in the dermis of the wild-type mouse (12 weeks old) with E-BMT in the skin specimens (HIS, $n = 6$). Original magnifications, $\times 400$ (A).

experiments, toluidine blue dye was added to the suspension of GFP-BMCs, allowing us to monitor successful E-BMT by blue color staining of the embryonic skin (Figure 1B). Within 30 minutes after E-BMT, successful distribution of the transplanted BMCs to the fetal skin was confirmed by observing GFP⁺ cells on the surface of the fetal skin by fluorescence stereoscopic microscopy as well as visual observation of blue color (Figure 1C).

Successful Engraftment of the GFP-BMCs in Hematopoietic Tissues of Mice with E-BMT

Successful long-term engraftment of the GFP-BMCs by E-BMT was confirmed by demonstration of hematopoietic chimerism of the donor and recipient cells in the BM and other hematopoietic tissues at 12 weeks after birth. Specifically, hematopoietic tissues, including BM, spleen, thymus, Peyer's patch, and lymph nodes, were shown to have an engraftment of cells derived from GFP-BMCs (Figure 2A). Fluorescence-activated cell sorting (FACS) analysis indicated that CD45⁺ hematopoietic cells derived from E-BMT accounted between 0.1% and 0.7% of the total BMC population after a single-dose of E-BMT with 1.0×10^6 GFP-BMCs in five individual mice (Figure 2, A–C). On the other hand, no GFP-BMCs were detected at 12 weeks after BMT with GFP-BMCs in adult mice

(Figure 2C). These findings suggest induction of immune tolerance against GFP protein by E-BMT.

Humoral and Cellular Immune Tolerance Induction against GFP by E-BMT

Previous studies have indicated that GFP protein is an intolerable immunogenic antigen that evokes both cellular and humoral immune response.²⁵ We then evaluated whether E-BMT of congenic GFP-BMCs can induce immune tolerance against GFP. First, E-BMT mice with 1.0×10^6 of congenic GFP-BMCs were transplanted with the skin from a congenic GFP-transgenic mouse (C57BL/6 background). Successful engraftment of the transplanted GFP-containing mouse skin was observed in all mice with E-BMT (Figure 3A–C), whereas the transplanted skin was rejected within 5 weeks after engraftment in all recipient mice without E-BMT (Figure 3C). Induction of anti-GFP antibodies, which was observed in all naïve mice with GFP skin transplantation, was completely inhibited by E-BMT (Figure 3D). Furthermore, cytotoxic immune reactivity of the thymic cells against cells harboring GFP was not detected by ⁵¹Cr release assay in E-BMT mice (Figure 3E). These data clearly demonstrate that E-BMT in fetal mice with congenic GFP-BMCs is sufficient to induce both humoral and cellular immune

tolerance against GFP. Secondly, we compared the potential for immune tolerance induction against GFP between E-BMT and neonatal subcutaneous transplantation with various GFP-expressing cells, such as BMCs, fibroblasts, and mesenchymal stem cells. None of these cells could induce immune tolerance against GFP by postnatal subcutaneous transplantation, subsequently resulting in immunological rejection of the transplanted GFP mouse skin (data not shown), as well as in raising anti-GFP antibodies (Figure 3F).

Skin-Homing BMCs Provide BMDFs in Uninjured Skin after Birth

Next, we searched for evidence of BMDFs in the skin of E-BMT mice after birth. Fluorescence stereoscopic microscopy examination showed that numerous GFP⁺ cells were dispersed over the entire skin of 12-week-old mice with E-BMT (Figure 4A). These GFP⁺ cells were scattered within the dermal structures, as shown in histological sections of the skin (Figure 4B). We then examined the expression of fibroblast marker proteins on the transplanted BMC-derived cells in the skin of 12-week-old mice with E-BMT. Immunohistochemical examination revealed that some of the GFP⁺ cells showed overlapping staining with antibodies against type I collagen (Figure 4C), fibronectin (Figure 4D), and vimentin (Figure 4, E–G). The GFP⁺ cells expressing fibroblast marker proteins were intermingled with GFP[−] fibroblasts particularly around hair follicle structures in the skin (Figure 4, F and G), but less in the region of the dermo-epidermal junction (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

To characterize the GFP⁺ cells expressing fibroblast marker proteins, dermal fibroblasts of the skin of 12-week-old mice with E-BMT were cultured. The GFP⁺ cells were morphologically indistinguishable from GFP[−] fibroblasts in culture (Figure 5A). Immunofluorescent staining clearly showed that the cultured GFP⁺ cells also expressed fibroblast marker proteins, including type I collagen, fibronectin, vimentin, and type VII collagen (Figure 5A).

After isolation of a pure GFP-positive population of cultured cells by a fluorescence-activated cell sorter (FACS), mRNA expression profiles for fibroblast markers of the sorted GFP⁺ cells were compared with fibroblasts and BMCs obtained from GFP-transgenic mice by RT-PCR. Identical profiles of the mRNA expression were observed between fibroblasts and the sorted GFP⁺ cells, whereas BMCs did not show any mRNA expression of the fibroblast marker genes (Figure 5B). Quantitative analysis

of the sorted GFP⁺ fibroblasts showed that ~0.5% of the cells were BMDFs in cultures of fibroblasts from six different mice with E-BMT (Figure 5C). In addition, we evaluated the percentage of the GFP-positive/vimentin-positive cells in the total vimentin-positive cell population in the dermis of the mice (12 weeks old) with E-BMT. After evaluation of six different, randomly selected microscopic fields of the skin specimens, ~0.3% of the vimentin-positive cells were also shown to be GFP-positive. Collectively, BMDFs were estimated to represent ~0.5% of the dermal fibroblast population in the skin of E-BMT mice.

E-BMT Improves Survival and Lessens the Severity of Skin Phenotype of DEB Mice by Providing BMDFs to the Skin

Finally, we evaluated the therapeutic potential of E-BMT to ameliorate pathogenic phenotypes of genetic skin diseases with fibroblast dysfunction by providing immunologically tolerated functional BMDFs to the skin. For this purpose, we performed E-BMT on DEB model mice with absent expression of type VII collagen, an adhesion molecule synthesized by both dermal fibroblasts and epidermal keratinocytes. E-BMT to the DEB mouse embryos prevented their neonatal death, which usually occurs within the first 2 days of birth (Figure 6A). Chimerism with GFP⁺ cells was also confirmed in the BMCs of the DEB mice with E-BMT (Figure 6B). Careful examination of the DEB mice with E-BMT revealed that these mice survived until 17 to 19 days of age, although none of them survived beyond 3 weeks (Figure 6C). RT-PCR analysis showed that type VII collagen expression was clearly detectable in the skin of DEB mice with E-BMT of GFP-transgenic BMCs, but not in the skin of DEB mice without E-BMT (Figure 6D). Immunohistochemical examination showed that type VII collagen, which is normally expressed in the basement membrane region of the dermo-epidermal junction and around the hair follicles of the skin (Figure 6E), but lost in the DEB mouse skin (Figure 6F), was restored in the vicinity of dermal GFP⁺ cells, possibly BMDFs. This observation was particularly evident in the follicular basement membrane region of the skin of DEB mice with E-BMT (Figure 6, G–P). However, dermo-epidermal separation, characteristic of DEB neonatal mice (Figure 6Q), was lessened but not entirely reversed in the DEB mice with E-BMT (Figure 6R).

Figure 6. E-BMT ameliorates defects in DEB mice after birth. **A:** Relatively long survival of the DEB mice with E-BMT. On the left side is a DEB mouse without E-BMT that died right after the delivery and on the right side is a DEB mouse with E-BMT (white arrow) that survived the neonatal period. **B:** BM showed a chimerical condition with GFP⁺ cells derived from E-BMT. **C:** Comparison of the survival of DEB mice with or without E-BMT. Note that significant improvement in survival after birth was obtained in the mice with E-BMT ($P < 0.01$). **D:** RT-PCR analysis for type VII collagen mRNA expression in the skin of GFP-transgenic mice (lane a), DEB mice with E-BMT of GFP-transgenic BMCs (lane b), and DEB mice without E-BMT (lane c). **E–H:** Immunostaining for type VII collagen in the skin of normal mice (E), DEB mice (F), and DEB mice with E-BMT of GFP-transgenic BMCs (G, H). Low magnification (G, in the white circle), middle magnification (H), and high magnification (I–L) indicate type VII collagen expression, particularly along the hair follicle structure in association with GFP⁺ BMDFs. **I:** 4,6-Diamidine-2-phenylindole dihydrochloride staining. **J:** GFP fluorescence. **K:** Type VII collagen staining. **L:** Merger of I–K. **M** and **N:** Keratin 5 expression in the follicular keratinocytes beside the BMDFs in the serial skin section compared to that of I–N. **M:** Keratin 5 staining. **N:** Merger of M and N. **O** and **P** indicate type VII collagen expression clearly around the hair follicle structure. H&E staining of the skin sections from DEB mice after birth with no E-BMT (Q), and with E-BMT (4 weeks after E-BMT) (R). White arrows indicate GFP⁺ BMDFs. Arrowheads indicate type VII collagen. Asterisks indicate dermo-epidermal separation. Original magnifications: ×80 (B); ×200 (E, H); ×240 (F); ×400 (E–G, I–P); ×100 (Q, R).

Discussion

In this study, we have demonstrated, for the first time, that adult BMC transplantation into the fetal circulation via the vitelline vein at embryonic day 13 can provide a significant number of BMDFs into the skin under physiological conditions, and that these BMDFs clearly persist in the skin beyond birth. Transfer of cultured fibroblasts or BM-derived mesenchymal stem cells (in amounts more than 0.5×10^6) to the embryonic circulation via the vitelline vein always resulted in embryonic death (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), possibly attributable to emboli in the microcirculation reflecting the cohesive nature of the cultured mesenchymal cells. These observations imply an apparent advantage of BMCs as compared to cultured fibroblasts or mesenchymal stem cells for embryonic transfer into circulation to generate *de novo* dermal fibroblasts.

BMDFs were not identified either in the transplanted BMC population or in the skin of the E-BMT mouse immediately after birth (data not shown), suggesting that progenitors of the BMDFs exist in the transplanted BM, which then migrate to the uninjured skin, and require appropriate matrix stimulation in the growing skin to become fibroblasts. Fetal mouse skin undergoes rapid growth in conjunction with mesenchymal maturation, processes in which stem cells can differentiate to adipose tissue, smooth muscle cells, and fibroblasts in the dermal matrix.^{26,27} Because BM has been shown to contain mesenchymal stem cells,^{28–30} transplanted BMCs in the fetal circulation may induce migration of the BM mesenchymal stem cells to the skin with recruiting signals derived from the growing skin.

In this context, it should be noted that E-BMT provided BMDFs to the skin preferentially around hair follicle structures of in both normal and DEB mice. In conjunction with the observation that BMDFs were rarely detected in esophageal lamina propria of normal mice with E-BMT (a few BMDFs were detected in only 1 microscopic field examined of 100 esophageal histological sections; see Supplemental Figure S3 at <http://ajp.amjpathol.org>), we speculate that the developmental stage and/or the growth stage of hair follicles may specifically determine expression of chemoattractants to recruit circulating BMDF progenitor/stem cells to the follicular regions in the skin.

Recently, we reported that bone morphogenic protein-2 (BMP-2) stimulation in muscle tissue recruits BM-derived circulating mesenchymal stem/progenitor cells to form ectopic bone.³¹ Because BMP-2 has also been shown to contribute to fetal skin development,³² this morphogen may also have a role in recruiting circulating mesenchymal stem/progenitor cells to the fetal skin to generate BMDFs after birth. Further investigation is necessary to disclose the precise mechanisms that generate BMDFs in growing, uninjured skin by E-BMT.³³

Our results presented in this report also suggest the possibility that E-BMT may be a therapeutic option for heritable skin diseases that are caused by genetic dysfunction of skin fibroblasts. To assess this possibility, we performed E-BMT to evaluate the therapeutic efficacy on

the mouse model of DEB, characterized by blistering and ulcerations of the skin because of dermo-epidermal separation as a result of homozygous ablation of the type VII collagen gene, *Col7a1*.³⁴ This mouse model recapitulates the clinical, genetic, histopathological, and ultrastructural features of human recessively inherited HS-RDEB. Type VII collagen is physiologically located in the basement membrane zone of the skin and the esophagus, and it secures adhesion of the corresponding epithelial tissues to the underlying mesenchyme through formation of anchoring fibrils.^{35–37} Because of decreased or absent type VII collagen, patients with DEB suffer from severe burn-like skin lesions, such as blisters, ulcers, and extensive scarring, as a result of minor external trauma throughout their entire life.³⁸ The severe scar formation of the skin is also associated with a high risk of squamous cell carcinoma in patients with RDEB.^{39,40} In some cases, swallowing of solid food may induce separation of the epithelial mucosa of the esophagus and repeated formation of esophageal ulcers may eventually lead to scar-induced constrictions, resulting in difficulty in eating. Mice with DEB will also have a feeding difficulties from the birth on, because of oral blisters and ulcers, resulting in premature demise within a few days of birth.³⁴ Type VII collagen is known to be produced by both epidermal keratinocytes and dermal fibroblasts,^{17–19} and supplementation of normal fibroblasts to the lesional skin from patients with DEB transplanted on nude mice has been shown to rescue skin phenotypes by providing type VII collagen.²⁰

Here, we demonstrated that E-BMT to the DEB mouse embryos provides BMDFs expressing type VII collagen in the skin and ameliorates the phenotypic severity, at least in part, by providing type VII collagen to the skin. As demonstrated above, BMDFs were predominately in the areas adjacent to hair follicles and barely detectable in the interfollicular region of the dermo-epidermal junction (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Because their preferential migration to the areas surrounding hair follicles, localized dermo-epidermal separation was still evident in the DEB mice with E-BMT at 3 weeks after birth (Figure 6R). We could not observe fully developed, mature anchoring fibrils that are formed from type VII collagen at the basement membrane region, by transmission electron microscopy (see Supplemental Figure S4A at <http://ajp.amjpathol.org>). However, we observed fibrillar structures, which may represent immature anchoring fibrils, at localized portions of the cutaneous basement membrane zone of the skin of the DEB mice with E-BMT (see Supplemental Figure S4B at <http://ajp.amjpathol.org>). Based on these observations, coupled with significant improvement in the survival of the newborn DEB mice with E-BMT, we believe that BMDFs, while providing type VII collagen molecules to the cutaneous basement membrane zone, contributed to some extent to improvement of the disease phenotype in DEB mice.

All DEB mice with E-BMT died at ~3 weeks after birth, at the time they start feeding on solid food. The limited number of the transplanted BMCs in the embryo might not be sufficient to provide an adequate number of type VII collagen-producing fibroblasts to the esophagus to

allow eating solid food. Indeed, the relative number of BMDFs in the mouse esophagus elicited by E-BMT was less than one-hundredth of the number of BMDFs in the skin (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). Nevertheless, we believe that our data provide a future perspective for application of E-BMT to the patients with DEB *in utero* when prenatal diagnosis indicates COL7A1 mutations, with predicted severe phenotype.

BMT is a well-established medical intervention for both children and adults afflicted with severe hematopoietic diseases, such as leukemia. However, there are still difficulties in finding HLA-matched donors for BMT. Therefore, patients must undergo treatment by irradiation and/or myeloablative reagents, in combination with immune suppressive therapies to prevent immune rejection reaction and to avoid graft-versus-host disease, before BMT can be performed. On the other hand, during the first trimester of human gestation the fetus has been shown to accept allogenic antigens by introducing immunological tolerance with clonal deletion of the allogenic antigen-reacting lymphocytes in the thymus.⁴¹ During this time period, E-BMT without matching HLA to the recipient is expected to successfully engraft without the need of myeloablative or immunosuppressive regimen.⁴² E-BMT in this time period can also be expected to introduce immune tolerance to a molecule that is expressed in the donor cells but not in the fetus because of genetic mutations.

Our results suggest that E-BMT can be an effective therapeutic option not only by providing functional fibroblasts to the lesional tissues, such as the skin, but also by introducing immune tolerance against the exogenously provided molecules (GFP) expressed in the donor cells, thus resulting in long-term survival of the GFP-expressing BMDFs in the recipient skin. On the other hand, subcutaneous transfer of various GFP⁺ cells, including BMCs and mesenchymal stem cells, failed to generate immune tolerance against GFP, demonstrating an advantage of E-BMT at least for tolerance induction against exogenous immunogenic molecules.

Our study also suggests that a limited number of BMCs may be sufficient to introduce immune tolerance against the molecules initially absent in the recipients if E-BMT is performed at the appropriate time frame in gestation with HLA-matched donor cells. A recent animal study suggested that allogenic BMCs may have short survival after birth as compared to congenic BMCs, even by E-BMT.¹² Other experimental and clinical studies have shown, however, that successful renal transplantation from HLA-mismatched donors can be achieved when combined with transplantation of hematopoietic stem cells from the same donors by nonmyeloablative conditioning, resulting in discontinuation of all immunosuppressive therapies afterward.⁴³ The report of these clinical trials suggested that initial transient BM chimerism of the recipient hematopoietic tissues with transplanted donor hematopoietic stem cells may be sufficient to induce central and/or peripheral immune tolerance to ensure stable maintenance of the transplanted allogenic kidney. In this context, E-BMT may introduce immune tolerance against type VII collagen in DEB patients who initially lack this

protein because of genetic mutations in the COL7A1 gene. If this is indeed the case, E-BMT may be established as an essential therapeutic option for severe HS-RDEB patients *in utero* to allow them to receive extensive gene-, cell-, or protein-based molecular therapies to cure the disease after birth.²³

Concerning the technical aspect of E-BMT, some human inherited diseases, such as hematological disorders (eg, Fanconi's anemia and thalassemia), immunological defects (eg, SCID), or metabolic diseases (eg, Hurler and Krabbe diseases) have already been clinically treated by E-BMT.¹³ In these diseases, E-BMT was shown to preserve the organ function. If BMC transplantation is performed postnatally, radiation therapy, intensive immunosuppression, and myeloablation have to be used to minimize the risk of rejection or graft-versus-host disease. For these reasons, we believe that E-BMT may have a rational advantage for treatment of EB patients as compared to postnatal BMT with immunosuppressive regimen. Such postnatal immunosuppressive procedures potentially have a high risk to induce severe cutaneous, and possibly systemic, infection in EB patients with multiple skin ulcers over the entire body surface.

E-BMT with self-BMCs transduced with a therapeutic gene would seem to be an ideal option for future embryonic gene therapy toward genetic skin diseases, provided that self-BMCs can be obtained from the fetus. Further continuous efforts of basic and clinical studies are required to establish E-BMT as an essential therapeutic strategy for currently intractable genetic diseases, such as DEB.

References

1. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002, 110:341–350
2. Ishii G, Sangai T, Oda T, Aoyagi Y, Hasebe T, Kanomata N, Endoh Y, Okumura C, Okuhara Y, Magae J, Emura M, Ochiya T, Ochiai A: Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem Biophys Res Commun* 2003, 309:232–240
3. Brittan M, Hunt T, Jeffery R, Poulson R, Forbes SJ, Hodivala-Dilke K, Goldman J, Alison MR, Wright NA: Bone marrow derivation of pericyptal myofibroblasts in the mouse and human small intestine and colon. *Gut* 2002, 50:752–757
4. Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, Poulson R, Hodivala-Dilke K, Alison MR, Wright NA: Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. *Stem Cells* 2003, 21:514–520
5. Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH: Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004, 113:243–252
6. Ogawa M, LaRue AC, Drake CJ: Hematopoietic origin of fibroblasts/myofibroblasts: its pathophysiologic implications. *Blood* 2006, 108:2893–2896
7. LaRue AC, Masuya M, Ebihara Y, Fleming PA, Visconti RP, Minamiguchi H, Ogawa M, Drake CJ: Hematopoietic origins of fibroblasts: I. In vivo studies of fibroblasts associated with solid tumors. *Exp Hematol* 2006, 34:208–218
8. Opaletnik SR, Davidson JM: Fibroblast differentiation of bone marrow-derived cells during wound repair. *FASEB J* 2005, 19:1561–1563
9. Ishii G, Sangai T, Sugiyama K, Ito T, Hasebe T, Endoh Y, Magae J, Ochiai A: In vivo characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions. *Stem Cells* 2005, 23:699–706

10. Flake AW: In utero transplantation of haemopoietic stem cells. *Best Pract Res Clin Haematol* 2001, 14:671-683
11. Javazon EH, Merchant AM, Danzer E, Flake AW: Reconstitution of hematopoiesis following intrauterine transplantation of stem cells. *Methods Mol Med* 2005, 105:81-94
12. Peranteau WH, Endo M, Adibe OO, Flake AW: Evidence for an immune barrier after in utero hematopoietic-cell transplantation. *Blood* 2007, 109:1331-1333
13. Troeger C, Surbek D, Schoberlein A, Schatt S, Dudler L, Hahn S, Holzgreve W: In utero haematopoietic stem cell transplantation. Experiences in mice, sheep and humans. *Swiss Med Wkly* 2006, 136:498-503
14. Briggaman RA, Wheeler Jr CE: The epidermal-dermal junction. *J Invest Dermatol* 1975, 65:71-84
15. Lunstrum GP, Kuo HJ, Rosenbaum LM, Keene DR, Glanville RW, Sakai LY, Burgeson RE: Anchoring fibrils contain the carboxyl-terminal globular domain of type VII procollagen, but lack the amino-terminal globular domain. *J Biol Chem* 1987, 262:13706-13712
16. Sakai LY, Keene DR, Morris NP, Burgeson RE: Type VII collagen is a major structural component of anchoring fibrils. *J Cell Biol* 1986, 103:1577-1586
17. Stanley JR, Rubinstein N, Klaus-Kovtun V: Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. *J Invest Dermatol* 1985, 85:542-545
18. Ryyanen J, Sollberg S, Parente MG, Chung LC, Christiano AM, Uitto J: Type VII collagen gene expression by cultured human cells and in fetal skin. Abundant mRNA and protein levels in epidermal keratinocytes. *J Clin Invest* 1992, 89:163-168
19. Woodley DT, Briggaman RA, Gammon WR, O'Keefe EJ: Epidermolysis bullosa acquisita antigen is synthesized by human keratinocytes cultured in serum-free medium. *Biochem Biophys Res Commun* 1985, 130:1267-1272
20. Woodley DT, Krueger GG, Jorgensen CM, Fairley JA, Atha T, Huang Y, Chan L, Keene DR, Chen M: Normal and gene-corrected dystrophic epidermolysis bullosa fibroblasts alone can produce type VII collagen at the basement membrane zone. *J Invest Dermatol* 2003, 121:1021-1028
21. Woodley DT, Remington J, Huang Y, Hou Y, Li W, Keene DR, Chen M: Intravenously injected human fibroblasts home to skin wounds, deliver type VII collagen, and promote wound healing. *Mol Ther* 2007, 15:628-635
22. Goto M, Sawamura D, Ito K, Abe M, Nishie W, Sakai K, Shibaki A, Akiyama M, Shimizu H: Fibroblasts show more potential as target cells than keratinocytes in COL7A1 gene therapy of dystrophic epidermolysis bullosa. *J Invest Dermatol* 2006, 126:766-772
23. Wong T, Gammon L, Liu L, Mellerio JE, Dopping-Hepenstal PJ, Pacy J, Elia G, Jeffery R, Leigh IM, Navsaria H, McGrath JA: Potential of fibroblast cell therapy for recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 2008 Apr. 3 [Epub ahead of print]
24. Chen M, Woodley DT: Fibroblasts as target cells for DEB gene therapy. *J Invest Dermatol* 2006, 126:708-710
25. Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D: Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther* 1999, 6:1305-1312
26. Van Exan RJ, Hardy MH: The differentiation of the dermis in the laboratory mouse. *Am J Anat* 1984, 169:149-164
27. Breathnach AS: Development and differentiation of dermal cells in man. *J Invest Dermatol* 1978, 71:2-8
28. Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997, 276:71-74
29. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 1999, 284:143-147
30. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002, 418:41-49
31. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y: Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. *Biochem Biophys Res Commun* 2007, 354:453-458
32. Stelnicki EJ, Longaker MT, Holmes D, Vanderwall K, Harrison MR, Lardman C, Hoffman WY: Bone morphogenetic protein-2 induces scar formation and skin maturation in the second trimester fetus. *Plast Reconstr Surg* 1998, 101:12-19
33. Ebihara Y, Masuya M, Larue AC, Fleming PA, Visconti RP, Minamiguchi H, Drake CJ, Ogawa M: Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. *Exp Hematol* 2006, 34:219-229
34. Heinonen S, Mannikko M, Klement JF, Whitaker-Menezes D, Murphy GF, Uitto J: Targeted inactivation of the type VII collagen gene (Col7a1) in mice results in severe blistering phenotype: a model for recessive dystrophic epidermolysis bullosa. *J Cell Sci* 1999, 112:3641-3648
35. Bächinger HP, Morris NP, Lunstrum GP, Keene DR, Rosenbaum LM, Compton LA, Burgeson RE: The relationship of the biophysical and biochemical characteristics of type VII collagen to the function of anchoring fibrils. *J Biol Chem* 1990, 265:10095-10101
36. Uitto J, Chung-Honet LC, Christiano AM: Molecular biology and pathology of type VII collagen. *Exp Dermatol* 1992, 1:2-11
37. Burgeson RE: Type VII collagen, anchoring fibrils, and epidermolysis bullosa. *J Invest Dermatol* 1993, 101:252-255
38. Horn HM, Tidman MJ: The clinical spectrum of dystrophic epidermolysis bullosa. *Br J Dermatol* 2002, 146:267-274
39. Yamada M, Hatta N, Sogo K, Komura K, Hamaguchi Y, Takehara K: Management of squamous cell carcinoma in a patient with recessive-type epidermolysis bullosa dystrophica. *Dermatol Surg* 2004, 30:1424-1429
40. Pourreyaon C, Cox G, Mao X, Volz A, Baksh N, Wong T, Fassihi H, Arita K, O'Toole EA, Ocampo-Candiani J, Chen M, Hart IR, Bruckner-Tuderman L, Salas-Alanis JC, McGrath JA, Leigh IM, South AP: Patients with recessive dystrophic epidermolysis bullosa develop squamous-cell carcinoma regardless of type VII collagen expression. *J Invest Dermatol* 2007, 127:2438-2444
41. Billingham RE, Brent L, Medawar PB: 'Actively acquired tolerance' of foreign cells 1. *Transplantation* 2003, 76:1409-1412
42. Flake AW, Zanjani ED: In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. *Blood* 1999, 94:2179-2191
43. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, Shaffer J, Preffer FI, Ding R, Sharma V, Fishman JA, Dey B, Ko DS, Hertl M, Goes NB, Wong W, Williams Jr WW, Colvin RB, Sykes M, Sachs DH: HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008, 358:353-361