Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（雑誌）

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Fujita Y, Yoshioka N, Abe R, Murata J, Hoshina D, Mae H, Shimizu H	Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis.	J Am Acad Dermatol			in press
Fujita Y, Abe R, Inokuma D, Sasaki M, Hoshina D, Natsuga K, Nishie W, McMillan JR, Nakamura H, Shimizu T, Akiyama M, Sawamura D, Shimizu H	Bone marrow transplantation restores epidermal basement membrane protein expression and rescues epidermolysis bullosa model mice.	Proc Natl Acad Sci USA	107	14345-50	2010

Abe R, Yamagishi SI, Fujita Y, Hoshina D, Sasaki M, Nakamura K, Matsui T, Shimizu T, Bucala R, Shimizu H	Topical application of anti-angiogenic peptides based on pigment epithelium-derived factor can improve psoriasis	J Dermatol Sci	57	183-91	2010
Shinkuma S, Akiyama M, Inoue A, Aoki J, Natsuga K, Nomura T, Arita K, Abe R, Ito K, Nakamura H, Ujiie H, Shibaki A, Suga H, Tsunemi Y, Nishie W, Shimizu H	Prevalent LIPH founder mutations lead to loss of P2Y5 activation ability of PA-PLA1alpha in autosomal recessive hypotrichosis.	Hum Mutat	31	602-10	2010
Hamasaka A, Yoshioka N, Abe R, Kishino S, Umezawa K, Ozaki M, Todo S, Shimizu H	Topical application of dehydroxymethylepoxyquinomicin improves allergic inflammation via NF-kappaB inhibition.	J Allergy Clin Immunol	126	400-3	2010

Nomura Y, Akiyama M, Nomura T, Nemoto-Hasebe I, Abe R, McLean WH, Shimizu H	Chromosome 11q13.5 variant: No association with atopic eczema in the Japanese population.	J Dermatol Sci	59	210-2	2010
Osawa R, Konno S, Akiyama M, Nemoto-Hasebe I, Nomura T, Nomura Y, Abe R, Sandilands A, McLean WH, Hizawa N, Nishimura M, Shimizu H	Japanese-Specific Filaggrin Gene Mutations in Japanese Patients Suffering from Atopic Eczema and Asthma.	J Invest Dermatol	130	2834-6	2010
Iitani MM, Abe R, Yanagi T, Hamasaka A, Tateishi Y, Abe Y, Ito M, Kondo T, Kubota K, Shimizu H.	Aleukemic leukemia cutis with extensive bone involvement.	J Am Acad Dermatol	63	539-41	2010

Takahashi T, Matsumoto S, Matsushita M, Kamachi H, Tsuruga Y, Kasai H, Watanabe M, Ozaki M, Furukawa H, Umezawa K, Todo S	Donor pretreatment with DHMEQ improves islet transplantation.	J Surg Res	163	e23-34	2010
Tomioka N, Morita K, Kobayashi N Tada M, Itoh T, Saitoh S, Kondo M, Takahashi N, Kataoka A, Nakanishi K, Takahashi M, Kamiyama T, Ozaki M, Hirano T, Todo S.	Array comparative genomic hybridization analysis revealed four genomic prognostic biomarkers for primary gastric cancers.	Cancer Genet Cytogenet	201	6-14	2010
Kaneko H, Suzutani T, Aoki K, Kitaichi N, Ishida S, Ishiko H, Ohashi T, Okamoto S, Nakagawa H, Hinokuma R,	Epidemiological and virological features of epidemic keratoconjunctivitis due to new human adenovirus type 54 in Japan.	Br J Ophthalmol			in press

Asato Y, Oniki S, Hashimoto T, Iida T, Ohno S.					
Iwata D, Kitamura M, Kitaichi N, Saito Y, Kon S, Namba K, Morimoto J, Ebihara A, Kitamei H, Yposhida K, Ishida S, Ohno S, Uede T, Onoe K, Iwabuchi K.	Prevention of experimental autoimmune uveoretinitis by blockade of osteopontin with small interfering RNA.	Exp Eye Res			In press
Mizuki N, Meguro A, Ota M, Ohno S, Shiota T, Kawagoe T, Ito N, Kera J, Okada E, Yatsu K, Song YW, Lee EB, Kitaichi N, Namba K, Horie Y, Takeno M, Sugita S,	Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci.	Nat Genet	42	703-6	2010

Mochizuki M, Bahram S, Ishigatsubo Y, Inoko H.					
Kitaichi N, Kitamura M, Namba K, Ishida S, Ohno S	Elevation of surfactant protein D, a pulmonary disease biomarker, in the sera of uveitis patients with sarcoidosis.	Jpn J Ophthalmol	54	81-4	2010

Ⅳ. 研究成果の刊行物・別刷

ORIGINAL ARTICLE

Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis

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Background: Life-threatening adverse drug reactions such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) sometimes start with clinical features of ordinary drug-induced skin reactions (ODSRs) and it may be difficult to make a correct diagnosis before severe mucocutaneous erosions occur. We have reported that serum granulysin levels are elevated (cut off: 10 ng/mL) in patients with SJS/TEN before generalized blisters form.

Objective: We sought to develop a rapid detection system for elevated serum granulysin to predict the progression from ODSRs.

Methods: Serum samples from 5 patients with SJS/TEN at 2 to 4 days before mucocutaneous erosions formed were analyzed. Sera from 24 patients with ODSRs and 31 healthy volunteers were also investigated as control subjects. We developed a rapid immunochromatographic assay for the detection of high-level serum granulysin using two different antigranulysin monoclonal antibodies.

Results: The immunochromatographic test showed positive results for 4 of 5 patients with SJS/TEN but only one patient of 24 with ODSR. The results correlated closely with those of enzyme-linked immunosorbent assays.

Conclusion: This novel test enables the prediction of SJS/TEN occurrence in patients even when only ODSRs have been observed. (J Am Acad Dermatol 10.1016/j.jaad.2010.04.042.)

Key words: adverse drug eruption; diagnostic test; granulysin; Stevens-Johnson syndrome; toxic epidermal necrolysis.

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by blister formation and widespread skin detachment.[1] In the early stage, SJS/TEN presents clinically as edematous papules or erythema multiforme–like target rashes, which are very similar to those of ordinary drug-induced skin reactions (ODSRs). Such a clinical course makes it difficult to reach a diagnosis of SJS/TEN in the early stage, and this results in high mortality. There is an urgent need for a method to distinguish between early-stage SJS/TEN and ODSR.

The method should be as fast as possible, because SJS/TEN usually occurs within a few days. Furthermore, the technique should be as clinically simple as possible, such as using immunochromatographic test strips that are available for the detection of influenza infections. Among several candidates for diagnostic markers, we examined soluble Fas ligand

Abbreviations used:

ODSR: ordinary drug-induced skin reaction
sFasL: soluble Fas ligand
SJS: Stevens-Johnson syndrome
TEN: toxic epidermal necrolysis

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(sFasL) and found that it is elevated in the sera of patients with SJS/TEN in the early stage, before mucocutaneous erosions appear.[2,3] It would be very useful to be able to predict the occurrence of SJS/TEN, but sFasL serum levels are too low (cut off: 100 pg/mL) for use in a rapid diagnostic device.

Chung et al[4] recently reported that granulysin is highly expressed in blisters of patients with SJS/TEN. We found that both serum granulysin and sFasL are higher in patients with early-stage SJS/TEN than in patients with ODSR.[5] Serum levels of granulysin are 100 times higher (cut off: 10 ng/mL) than those of sFasL. Based on these observations, we developed a rapid immunochromatographic assay for the detection of high-level serum granulysin to diagnose and predict the early stage of SJS/TEN.

CAPSULE SUMMARY

- Life-threatening adverse drug reactions sometimes start with ~~maculopapular rash~~. Therefore, it is difficult to diagnose them in the early stage.
- We recently found that serum granulysin levels are elevated in patients who afterward developed Stevens-Johnson syndrome and toxic epidermal necrolysis.
- In this report we developed a novel immunochromatographic test to detect high serum granulysin. With this device we can predict whether patients with ~~maculopapular rash~~ develop severe drug eruptions.

METHODS

Patients

SJS refers to cases with mucosal erosions and epidermal detachment of less than 10% of the body surface area, and TEN refers to those with more than 30% involvement. Disease onset in patients with SJS/TEN was defined as the day when the mucocutaneous or ocular lesion first eroded or ulcerated (day 1).[3] From multiple Japanese institutions, we obtained serum samples from 35 patients with SJS/TEN.[3] Of these, we investigated 5 patients whose sera had been collected before the diagnosis of SJS/TEN (day −2 to −4). The patient [T1] information is listed in Table I. Serum samples from patients with ODSR (n = 24) and healthy volunteers (n = 31) were also analyzed. Informed consent was obtained from all patients, and the procedures were approved by the Ethical Committee of the Hokkaido University Graduate School of Medicine, Sapporo, Japan.

Immunochromatographic assay

In the immunochromatographic test, a murine monoclonal antibody specific to human granulysin (RB1, MBL, Nagoya, Japan) was conjugated with microparticles and then placed on the glass membrane area of the test device in a dry state. Another granulysin monoclonal antibody (RC8, MBL) was immobilized on a nitrocellulose membrane to form a result line. Likewise, a control line was created by the immobilization of antimouse IgG. The granulysin in the serum sample specifically bound to the microparticles via RB1 and comigrated upward until the granulysin was sandwiched with the immobilized RC8, revealing a visible result line. The entire test procedure was completed within 15 minutes.

Enzyme-linked immunosorbent assay

The granulysin concentrations of the serum samples were measured with a sandwich-enzyme-linked immunosorbent assay as previously described.[6,7] In brief, 96-well flat-bottomed plates were coated with 5 mg/mL of RB1 antibody and stored overnight at 4°C. The plates were then washed and blocked with phosphate-buffered saline containing 0.1% Tween-20 (washing buffer) and blocked with 10% fetal bovine serum in washing buffer at room [Q3] temperature for 2 hours. The samples and standards (recombinant granulysin, R&D Systems, Minneapolis, MN) were incubated for 2 hours at room temperature. Then they were reacted with 0.1 mg/mL of biotinylated RC8 antibody for 1 hour. The plates were then treated with 0.2 mg/mL of horseradish-peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland) for 30 minutes at room temperature. The plates were incubated with tetramethylbenzidine substrate (Sigma, St Louis, MO) for 30 minutes at room temperature, and then ~~2N H_2SO_4~~ was added. [Q4] The optical density was measured at 450 nm using a microplate reader (Mithras LB940, Berthold Technologies, Thoiry, France).

RESULTS

We first applied diluted recombinant human granulysin protein to the immunochromatographic test strips, to confirm the threshold and reliability of the assay. Approximately 10 ng/mL of sample yielded a result line, and 3 repeated investigations brought the same results (Fig 1, *A*). [F1-4/C]

Based on this observation, we then applied serum samples to detect the elevated granulysin levels. Four of 5 SJS/TEN samples showed positive results (Fig 1, *B*). All the positive samples had elevated granulysin as detected by enzyme-linked immunosorbent assay analysis (30.35 ± 9.91 ng/mL, average ± SEM). The

Table I. Patient information

Patient No.	Age, y	Sex	Diagnosis	Affected skin area	Causative drug	Serum granulysin (d)
1	17	M	SJS	20%	Carbamazepine	52.1 (−3)
2	66	F	TEN	70%	Imatinib	14.2 (−3)
3	27	F	SJS	<10%	Unknown	42.2 (−4)
4	80	M	SJS	5%	Phenytoin	12.9 (−2)
5	25	F	SJS	Only mucosal lesions	Unknown	2.7 (−2)

F, Female; *M*, male; *SJS*, Stevens-Johnson syndrome; *TEN*, toxic epidermal necrolysis.

only sample with a negative result had granulysin at the normal level of 2.7 ng/mL. Conversely, one in 24 ODSRs samples and none of 31 healthy volunteers showed positive bands in this immunochromatographic test. The test showed a sensitivity of 80% and a specificity of 95.8% for SJS/TEN versus ODSRs. The results of the immunochromatographic test correlated closely with early diagnosis for SJS/TEN ($P = 1.02 \times 10^{-3}$, analyzed by Fisher exact probability test).

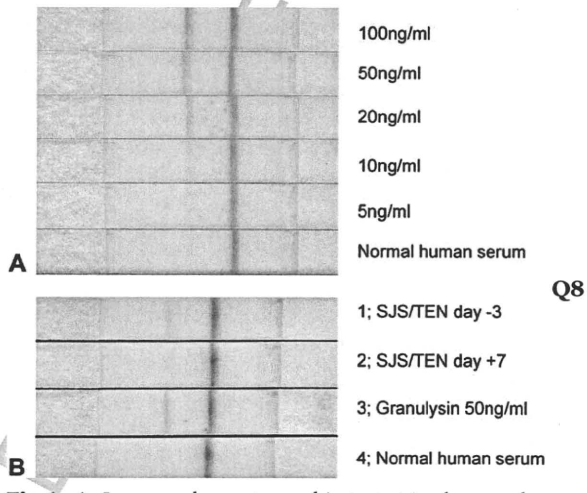

Fig 1. A, Immunochromatographic test strip detects elevated granulysin. *1 to 6*, Diluted recombinant granulysin is applied. ~~7~~, Normal human serum as negative control (1.4 ng/mL). Approximately 10 ng/mL of granulysin induces positive result. **B**, Detection of serum granulysin by immunochromatographic test. *1*, Serum taken from patient 1 with early Stevens-Johnson syndrome (*SJS*)/toxic epidermal necrolysis (*TEN*) at 3 days before blister formation. Although patient showed only edematous erythema and papules without mucous manifestations, serum granulysin was measured as 52.1 ng/mL afterward. *2*, Ten days after blister formation in same patient with SJS/TEN. No bands are observed, and serum granulysin has decreased to 5.7 ng/mL. *3*, Recombinant human granulysin as positive control. *4*, Normal human serum as negative control (3.5 ng/mL).

DISCUSSION

We succeeded in developing a rapid immunochromatographic test for the detection of high-level serum granulysin that puts our previous findings to practical use. Although 20% of the cases could be missed, it would be a useful adjunct in diagnosing SJS/TEN. It would not be necessary for every morbilliform drug eruption. We suggest that the test be applied when clinical findings hinting at SJS/TEN, such as target lesions, are seen. However, two biopsies should be done as soon as SJS/TEN are suspected, for hematoxylin-eosin ~~and frozen looking~~ for necrotic keratinocytes, which is another sensitive test.[8] If the results of either method are negative, careful daily and hourly monitoring of the patient for a few days should take place. Furthermore, to assess the severity of illness and to predict mortality, we should use the mathematical tool called SCORTEN that has been developed.[9]

Granulysin, a member of the saposin-like protein family of lipid-binding proteins, exhibits potent cytotoxicity against a broad panel of microbial targets, including tumor cells, transplanted cells, bacteria, fungi, and parasites, damaging negatively charged cell membranes.[10] Granulysin plays important roles in host defense against pathogens, and it induces apoptosis of target cells in a mechanism involving caspases and other pathways.[11] Chung et al[4] reported that granulysin was identified as the most highly expressed cytotoxic molecule in blisters of patients with SJS/TEN. Very recently, we showed that granulysin levels of sera from patients with SJS/TEN are significantly elevated before the development of skin detachment or mucosal lesions.[5] The elevated serum granulysin levels decrease rapidly within 5 days after disease onset. Such an elevation pattern is similar to that of sFasL.[3] When granulysin levels for patients with SJS/TEN in the early stage were compared with those levels for patients with ODSR and healthy control subjects, the differences were statistically significant.[5]

This novel test enables the early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that are otherwise indistinguishable from ODSRs.

REFERENCES

1. Roujeau JC, Kelly JP, Naldi L, Rzany B, Stern RS, Anderson T, et al. Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. N Engl J Med 1995;333:1600-7.
2. Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H, Shimizu H. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. Am J Pathol 2003;162:1515-20.
3. Murata J, Abe R, Shimizu H. Increased soluble Fas ligand levels in patients with Stevens-Johnson syndrome and toxic epidermal necrolysis preceding skin detachment. J Allergy Clin Immunol 2008;122:992-1000.
4. Chung WH, Hung SI, Yang JY, Su SC, Huang SP, Wei CY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. Nat Med 2008;14:1343-50.
5. Abe R, Yoshioka N, Murata J, Fujita Y, Shimizu H. Granulysin as a marker for early diagnosis of the Stevens-Johnson syndrome. Ann Intern Med 2009;151:514-5.
6. Ogawa K, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, et al. Granulysin in human serum as a marker of cell-mediated immunity. Eur J Immunol 2003;33:1925-33.
7. Saigusa S, Ichikura T, Tsujimoto H, Sugasawa H, Majima T, Kawarabayashi N, et al. Serum granulysin level as a novel prognostic marker in patients with gastric carcinoma. J Gastroenterol Hepatol 2007;22:1322-7.
8. Pereira FA, Mudgil AV, Rosmarin DM. Toxic epidermal necrolysis. J Am Acad Dermatol 2007;56:181-200.
9. Bastuji-Garin S, Fouchard N, Bertocchi M, Roujeau JC, Revuz J, Wolkenstein P. SCORTEN: a severity-of-illness score for toxic epidermal necrolysis. J Invest Dermatol 2000;115:149-53.
10. Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, et al. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol 2001;167:350-6.
11. Clayberger C, Krensky AM. Granulysin. Curr Opin Immunol 2003;15:560-5.

Bone marrow transplantation restores epidermal basement membrane protein expression and rescues epidermolysis bullosa model mice

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Attempts to treat congenital protein deficiencies using bone marrow-derived cells have been reported. These efforts have been based on the concepts of stem cell plasticity. However, it is considered more difficult to restore structural proteins than to restore secretory enzymes. This study aims to clarify whether bone marrow transplantation (BMT) treatment can rescue epidermolysis bullosa (EB) caused by defects in keratinocyte structural proteins. BMT treatment of adult collagen XVII (Col17) knockout mice induced donor-derived keratinocytes and Col17 expression associated with the recovery of hemidesmosomal structure and better skin manifestations, as well improving the survival rate. Both hematopoietic and mesenchymal stem cells have the potential to produce Col17 in the BMT treatment model. Furthermore, human cord blood CD34$^+$ cells also differentiated into keratinocytes and expressed human skin component proteins in transplanted immunocompromised (NOD/SCID/γ_c^{null}) mice. The current conventional BMT techniques have significant potential as a systemic therapeutic approach for the treatment of human EB.

hematopoietic stem cells | type XVII collagen

Bone marrow-derived cells, including hematopoietic stem cells and mesenchymal stem cells, have been reported to play a significant role in the recovery of various impaired organs (1–8). Although some papers have reported that "transdifferentiation" of circulating hematopoietic stem cells is an extremely rare event (9), previous reports have shown that expression of systemic enzymes and certain secreted factors can be recovered after bone marrow transplantation (BMT) (10).

Epidermolysis bullosa (EB) comprises a group of genodermatoses, which are caused by mutations in one of the genes encoding anchor proteins that stabilize the basement membrane zone (BMZ) of the skin and mucous membranes (11). Collagen XVII (COL17) is a transmembrane component of hemidesmosomal adhesion structures anchoring cells to the BMZ. *COL17* is the gene underlying non-Herlitz junctional EB in humans, a disorder that causes severe skin fragility, hair loss, growth retardation, and enamel hypoplasia (11). There is no effective treatment for EB other than palliative care. Gene-treated cultured autografting, reported by Mavilio et al. (12), is a promising therapeutic approach for junctional EB. However, its effects are limited to the area of application, in addition to the ethical and safety problems of using viruses for gene correction, even if the recent development of lentiviral vectors with favorable safety might be able to avoid the risks of gene augmentation with traditional retroviral constructs (13). Therefore, systemic and ethically safer therapies would be preferable.

Previous work reported that cells of human or murine origin do home to the skin, such as in graft-versus-host disease in humans (14) and epithelial progenitors in murine bone marrow (15). Our group reported that donor-derived keratinocytes could be identified at wound sites in a BMT model (16). This suggests that BMT techniques have the potential to provide functional keratinocytes over the entire skin surface. The current study investigates whether BM-derived cells can differentiate into donor-derived keratinocytes and subsequently produce detectable COL17 protein after BMT, with the ultimate goal of improving the clinical phenotype and contributing to long-term survival in our model mice.

Results

Donor BM-Derived Cells Express Col17 Protein in the BMZ in Recipient Mouse Skin. To investigate whether BM-derived keratinocytes can produce skin component proteins, we transplanted BM-derived cells of C57BL/6 mice expressing *human COL17* (*hCOL17*) driven by the keratin 14 promoter ($COL17^{m+/+,h+}$) into wild-type C57BL/6 mice in the first set of experiments. Detection of hCOL17 protein in the epithelized recipient skin would indicate that donor BM-derived cells had differentiated into keratinocytes. Immunohistochemical analysis revealed hCOL17 protein expression in the BMZ within the wounded area for four out of the eight BMT-treated C57BL/6 mice (Fig. 1*A*). RT-PCR analysis also showed evidence of *hCOL17* mRNA expression in five out of the eight treated mice (Fig. 1*B*).

We subsequently performed another BMT experiment: BM-derived cells from GFP$^+$ Tg mice were transplanted into COL17-humanized ($COL17^{m-/-,h+}$) mice (Fig. S1) (17). In this experimental pattern, BM-derived cells that differentiated into keratinocytes in the host mice were found to have the potential to produce mCol17 protein. BM-derived nonhematopoietic cells expressing GFP$^+$ CD45$^-$ were sparsely observed, accounting for $1.83 \pm 0.82\%$ ($n = 5$) of the basal layer cells (Fig. 1*C*). Aggregated GFP$^+$ cytokeratin$^+$ cells were also found in the basal cell layer (Fig. 1*D*). Epithelized skin areas in this experiment demonstrated mCol17 protein expression, although unwounded areas of the transplanted $COL17^{m-/-,h+}$ mice failed to express that protein (Fig. 1*E*). This mCol17 expression lasted at least 9 mo after wound formation in two out of the three investigated mice (Fig. S2). RT-PCR analysis also revealed the expression of *mCol17* mRNA in epithelized skin from four of the five transplanted mice, indicating that the BM-derived epidermal cells were able to express active *mCol17* (Fig. 1*F*).

Author contributions: Y.F., R.A., D.I., W.N., T.S., M.A., D.S., and H.S. designed research; Y.F., D.I., M.S., D.H., K.N., W.N., J.R.M., H.N., and D.S. performed research; K.N. and W.N. contributed new reagents/analytic tools; and Y.F., R.A., M.S., J.R.M., M.A., and H.S. analyzed data; and Y.F. and R.A. wrote the paper.

The authors declare no conflict of interest.

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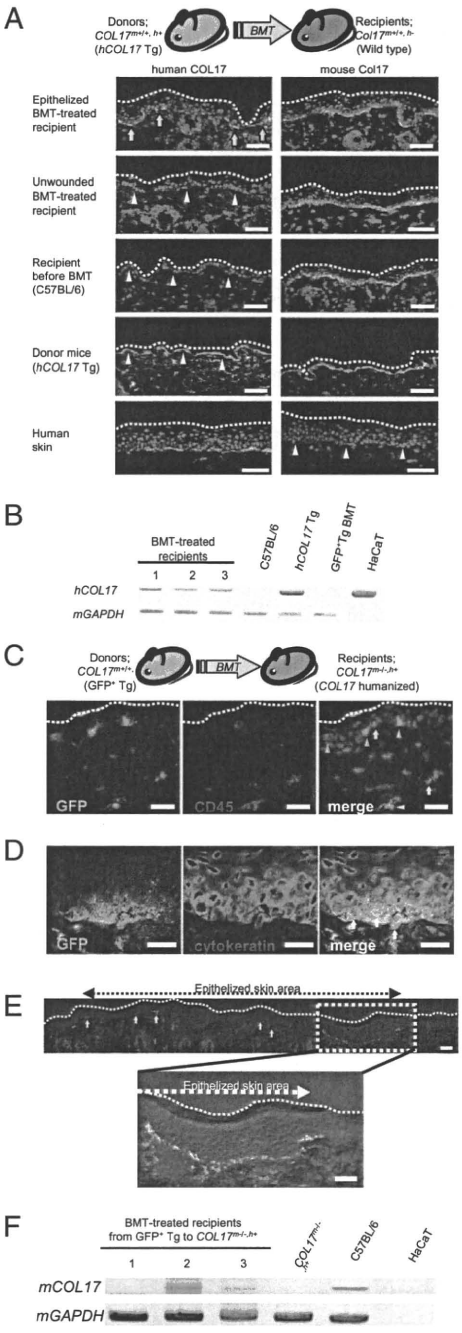

Fig. 1. BMT-induced donor cell-derived COL17 in the epithelized skin tissue. (*A*) The donor-derived hCOL17 expression is observed in the epithelized skin areas of BMT-treated C57BL/6 recipients (yellow arrows). Green: mCol17 (KT4.2) or hCOL17 (D20); red: nuclei; broken lines: skin surface; arrowheads: BMZ. (Scale bars: 50 μm.) (*B*) Representative RT-PCR analysis for *hCOL17* expression reveals positive bands in BMT-treated C57BL/6 recipients. All RNA samples were extracted from full-thickness skin biopsies, except for HaCaT from cultured cells. (*C*) Immunohistochemical analysis of the BMT-treated $COL17^{m-/-, h+}$ skin tissue demonstrates donor-derived GFP^+ $CD45^+$ blood cells (yellow arrowheads) and recipient-derived GFP^- $CD45^+$ cells (yellow arrow). Donor-derived GFP^+ $CD45^-$ (green arrowheads) cells are sporadically noted in the epidermis. (Scale bars: 20 μm.) (*D*) Aggregated donor-derived GFP^+ cells in the basal cell layer are noted, some of which also express cytokeratin (white arrows). These cells are thought to be donor-derived keratinocytes. (Scale bars: 20 μm.) (*E*) The skin of the recipients shows sporadic, linear deposition of mCol17 (arrows). The deposition is limited to the epithelized skin area with acanthosis. (*Upper*) The entire consolidated image. (*Lower*) Higher magnification. (Scale bars: 50 μm.) (*F*) RT-PCR analysis shows the recovery of *mCol17* mRNA in two out of three representative mice (lanes 2 and 3).

BM-Derived Cells Supply Deficient Col17 Protein in the Col17 Knockout EB Model Mice. Our group recently established non-Herlitz junctional EB model mice ($COL17^{m-/-}$) as a result of homozygous ablation of the Col17 gene (17). Unlike EB model mice reported by other researchers, our model has allowed us to obtain adult $COL17^{m-/-}$ mice that can be used for various therapeutic strategies (Fig. S3). We speculated that mCol17 protein would be reintroduced by administering BM-derived cells from BMT treatments into $COL17^{m-/-}$ EB model mice. We transplanted BM cells of the GFP^+ Tg mice into the $COL17^{m-/-}$ mice. All of the mice obtained hematopoietic chimeras (710. ± 4.0%, $n = 21$). Immunohistochemical analysis revealed sporadic GFP^+ cells in the basal cell layer of the epidermis, accounting for 1.08 ± 0.39% of the basal cells ($n = 11$). GFP^+ $CD45^-$ cells including cytokeratin$^+$ epidermal keratinocytes were also found (0.26% ± 0.08% of basal cells) (Fig. 2*A* and Fig. S4). Linear deposition of mCol17 along the BMZ, and GFP^+ cells above the mCol17 staining were observed, accounting for 14.7 ± 3.0% ($n = 11$) of the epithelized area (Fig. 2*B*). Also, a 180-kDa mCol17 protein was detected in Western blotting (Fig. 2*C*). One out of three mice showed positive mCol17 immunohistochemically in unwounded skin from the back (Fig. S5*A*). Eight out of nine BMT-treated mice showed positive *mCol17* mRNA in the epithelized skin tissues. Compared with unwounded skin, epithelized areas of skin tended to show *mCol17* mRNA expression more frequently (Fig. S5*B*). We also performed RT-PCR analysis on the epithelized areas of BMT-treated $COL17^{m-/-}$ mice from the epidermis and the dermis by detaching each side enzymatically; this revealed positivity only on the epidermal side (Fig. 2*D*). Next, we sorted GFP^+ cells from the single-cell suspension of epithelized epidermal cells. A portion of the suspended epidermal cells showed GFP (1.24 ± 0.12%, $n = 4$; Fig. S6), and mRNA expression specific to epidermal keratinocytes was detected from the extract of the GFP^+ epidermal cells. These cells also expressed *mCol17* mRNA in three out of the four investigated mice (Fig. 2*E*). To rule out the possibility that cell fusion was occurring between BM cells and original keratinocytes in the $COL17^{m-/-}$ mice, we performed FISH analysis in a sex-mismatched BMT model. Several fused cells with XXXY chromosomes in the same nucleus were found in the deep dermis of the epithelized skin (Fig. 2*F*). Conversely, no fused cells were found in the epidermis of the samples we investigated, whereas 50 of 1,793 basal cells (2.79%) showed donor-derived XY chromosomes. To investigate the restoration of COL17 expression and its effect on restoring normal BMZ structure, we performed electron microscopic analysis. In the BMT-treated $COL17^{m-/-}$ mice, a portion of the basal cells had mature hemidesmosomes (Fig. 2*G*). The average thickness of outer plaques of hemidesmosomes was 79.7 ± 3.2 nm in the wild-type mice, 45.1 ± 1.4 nm in the untreated $COL17^{m-/-}$ mice, and 61.1 ± 1.9 nm in the BMT-treated $COL17^{m-/-}$ mice ($P < 0.01$). To exclude the nonspecific effects of bone marrow infusion, a mixture of lineage$^+$ differentiated GFP^+ BM cells and lineage$^-$ $COL17^{m-/-}$ BM cells was transplanted into the $COL17^{m-/-}$ mice. No Col17 expression of mRNA and protein were detected in the epithelized skin ($n = 3$).

Col17 Knockout Mice Exhibit Less Severe Clinical Manifestations and Better Survival Prognosis After BMT than Untreated Mice. To investigate the change in vulnerability to friction in skin that resulted from the restoration of Col17, we rubbed the back of each mouse (18). The BMT-treated mice ($n = 6$) significantly showed formation of smaller erosions compared with the untreated mice ($n = 4$) (Fig. 3*A*). Although our $COL17^{m-/-}$ mice survived longer than previously reported EB models, only 12.5% of the mice survived to 1 mo, approximately half of which died within the following 3 mo (17). Surprisingly, 16 out of the 20 transplanted $COL17^{m-/-}$ mice survived to 100 d after BMT (transplanted on d 35 after birth), whereas only 7 out of the 17 untreated $COL17^{m-/-}$ mice survived to

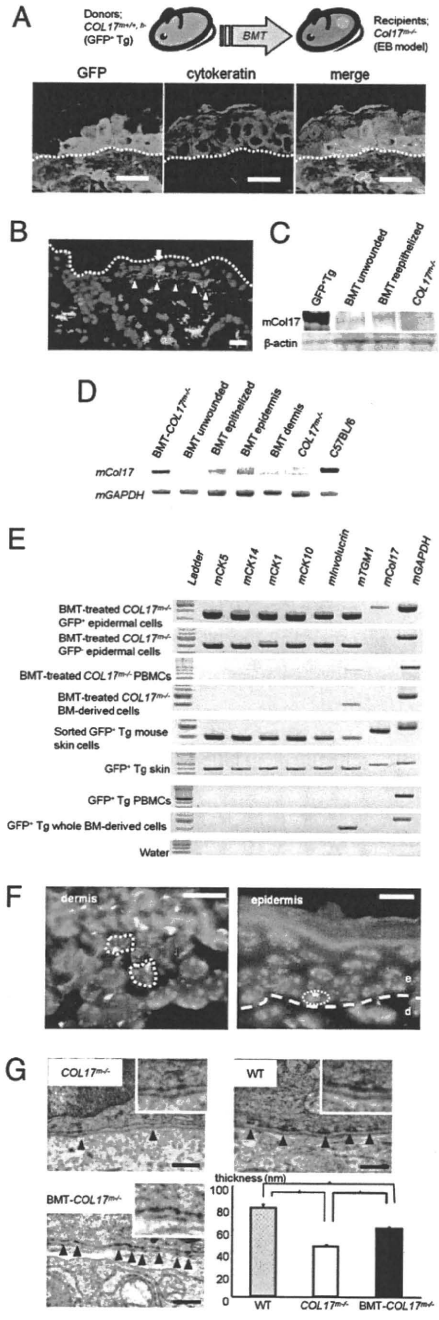

Fig. 2. BMT treatments induce functional mCol17 in $COL17^{m-/-}$ junctional EB model mice. (*A*) In the epithelized skin tissue of BMT-treated mice, a cluster of GFP^+ cytokeratin$^+$ basal cells is observed. Green: GFP; blue: nuclei; broken lines: skin surface. (Scale bars: 10 μm.) (*B*) Sporadic GFP^+ cells (green) are shown in the epithelized skin of the recipients (arrow). Furthermore, linear staining of mCol17 is detected in the BMZ (red, KT4.2, arrowheads). (Scale bars: 20 μm.) (*C*) Western blotting analysis reveals the expression of mCol17 in the epithelized area of the BMT-treated $COL17^{m-/-}$ mouse (lane 3), and a weak band is seen in unwounded skin of a BMT-treated $COL17^{m-/-}$ mouse (lane 2). β-actin: loading control. (*D*) The expression of *mCol17* is detected only in the epithelized skin and not in the unwounded skin area. Also, the expression is limited to the epidermis side of the epithelized skin. (*E*) The sorted GFP^+ single epidermal cells of BMT-treated $COL17^{m-/-}$ mice express various keratinocyte-specific mRNAs as well as *mCol17*. Sorted GFP^- cells express these mRNAs, other than that of mCol17. (*F*) No fused cells are apparent in the epidermis, although donor-derived XY cells are sparsely shown. Sporadic fused cells with XXXY chromosomes are observed in the deep dermis. Dashed circles indicate the border of the nucleus. e: epidermis; d: dermis. (Scale bars: 10 μm.) (*G*) The epithelized skin of $COL17^{m-/-}$ mice has hypoplastic hemidesmosomes with thin, poorly formed inner/outer plaques (arrowheads). In BMT-treated $COL17^{m-/-}$ mice, hemidesmosomes with mature plaques are seen. (Scale bars: 500 nm.) $^*P < 0.01$.

d 135 after birth. The survival outcomes were 73.7% for the transplanted group and 27.5% for the untreated group at d 200 after BMT (Fig. 3*B*). The BMT technique brought significant therapeutic benefits to the $COL17^{m-/-}$ EB model mice.

The untreated adult $COL17^{m-/-}$ EB model mice showed spontaneous erosions, ulcers, nail deformity, hair loss, and hair graying similar to those seen in human junctional EB patients lacking *COL17* (17). The erosions were especially severe in the genital regions (Fig. 3*C*). The BMT-treated $COL17^{m-/-}$ mice showed improvements to clinical manifestations, with fewer spontaneous erosions than for the untreated $COL17^{m-/-}$ mice and BMT-control $COL17^{m-/-}$ mice ($COL17^{m-/-}$ mice as donors). The improvements were particularly marked in the genital regions (Fig. S7).

Both Hematopoietic and Mesenchymal Stem Cells Contribute to the Expression of Col17. Because BM cells consist of various differentiated hematopoietic cells and stem cells, there is the question of which type of BM-derived stem cells produced the Col17 and caused clinical improvement in the $COL17^{m-/-}$ EB model mice. We obtained hematopoietic stem cells (HSCs) and multipotent mesenchymal stromal cells (MSCs) from GFP^+ Tg mice, following transplantation of each type of stem cell into $COL17^{m-/-}$ mice with whole $COL17^{m-/-}$ BM-derived cells as supporting cells (Fig. 4*A*). Four weeks after BMT, we confirmed partial chimerism ($37.0 \pm 13.7\%$, $n = 5$) of GFP in peripheral blood of BMT-treated mice with GFP^+ HSCs (HSC-BMT mice), whereas no GFP^+ peripheral blood cells were detected in BMT-treated mice with GFP^+ MSCs (MSC-BMT mice, $n = 4$) (Fig. S8). Immunohistochemical analysis revealed sparse GFP^+ cytokeratin$^+$ keratinocytes in the skin of the HSC- and MSC-BMT mice (Fig. 4*B*). Both HSCs and MSCs were found to have the potential to produce mCol17 as observed immunohistochemically; three out of five HSC-BMT mice and two out of four MSC-BMT mice showed positive mCol17 (Fig. 4*C*). RT-PCR analysis also demonstrated the expression of *mCol17* in both the HSC-BMT model (three out of five mice) and the MSC-BMT model (two out of four mice) (Fig. 4*D*). HSC-BMT mice showed better clinical manifestations than untreated $COL17^{m-/-}$ mice, whereas mice of the MSC-BMT model had a tendency to show more severe perianal erosions and hair loss (Fig. 4*E*).

Transplanted Human Cord Blood $CD34^+$ Cells Obtain a Keratinocyte-Like Phenotype and Produce Epidermal Component Proteins. Toward clinical applications of stem cell transplantation therapies in human EB patients, we investigated whether the human hematopoietic stem cells have the ability to supply structural proteins in the BMZ of the skin. A human-to-mouse xenogeneic transplantation model was investigated using NOD/SCID/γ_c^{null} (NOG) mice (19). Using immunohistochemistry, human cells that expressed human leukocyte antigen (HLA)-ABC could be seen, and pancytokeratin-positive cells were sporadically costained ($0.39 \pm 0.15\%$ of the basal cells, $n = 4$) (Fig. 5*A* and *B*), which indicates that these cells are donor cell-derived keratinocytes. In addition, sparse and intermittent hCOL17 was detected along the BMZ in two of seven treated mice (Fig. 5*C*). RT-PCR analysis surprisingly showed mRNA expression of several components of the normal human BMZ other than *hCOL17* (detected in six of seven treated mice), including *BPAG1* (four of seven), *plectin* (four of seven), *α6 integrin* (five of seven), *laminin β3* (two of seven), and *laminin γ2* (one out of seven) (Fig. 5*D*).

Discussion

Junctional EB is caused by mutations in the genes coding for structural proteins anchoring the skin to the underlying basal

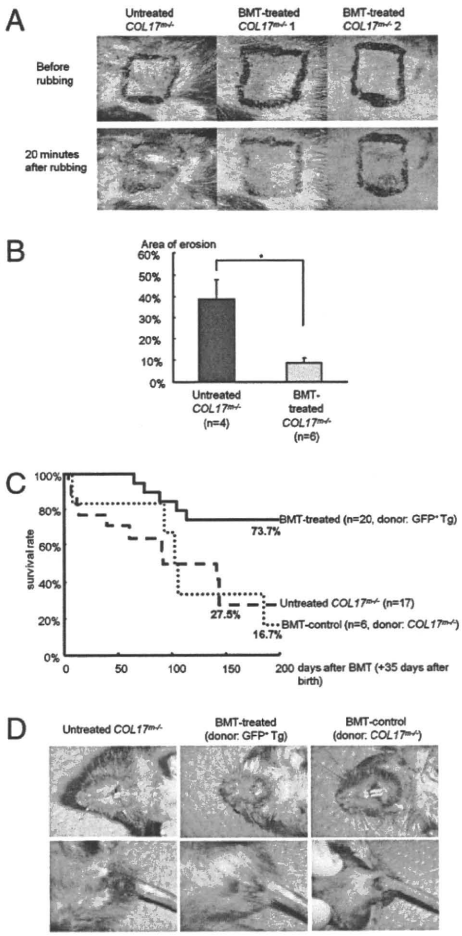

Fig. 3. BMT treatments in $COL17^{m-/-}$ junctional EB model mice change vulnerability to friction in the skin and induce better clinical conditions. (*A*) epithelized areas after erosion formation are investigated by rubber stress test. In untreated $COL17^{m-/-}$ mice, mild mechanical stimulus induces large erosions. Conversely, BMT-treated mice show less severe erosions. $*P < 0.05$. (*B*) The erosion area expressed as a percent of the rubbed area is measured for each group. Resistance of the skin to mechanical stimuli is significantly improved in the BMT-treated $COL17^{m-/-}$ mice. (*C*) Survival curves of BMT-treated and -untreated $COL17^{m-/-}$ mice from d 35 after birth (the day of BMT). $COL17^{m-/-}$ mice treated with BMT from $COL17^{m-/-}$ mice are shown as the BMT control mice; 73.7% of BMT-treated $COL17^{m-/-}$ mice could be expected to live over 200 d after BMT treatment vs. only 27.5% of untreated $COL17^{m-/-}$ mice and 16.7% of BMT control mice. ($P = 0.015$ for BMT-treated vs. untreated mice, $P = 0.021$ for BMT-treated vs. BMT control, and $P = 0.964$ for BMT control vs. untreated.). (*D*) Clinical manifestations at 90 d after BMT treatment (125 d after birth). Untreated $COL17^{m-/-}$ mice show moderate perioral erosions with crusts and anal erosions occurring spontaneously. In contrast, BMT-treated $COL17^{m-/-}$ mice show mild erosions in these areas.

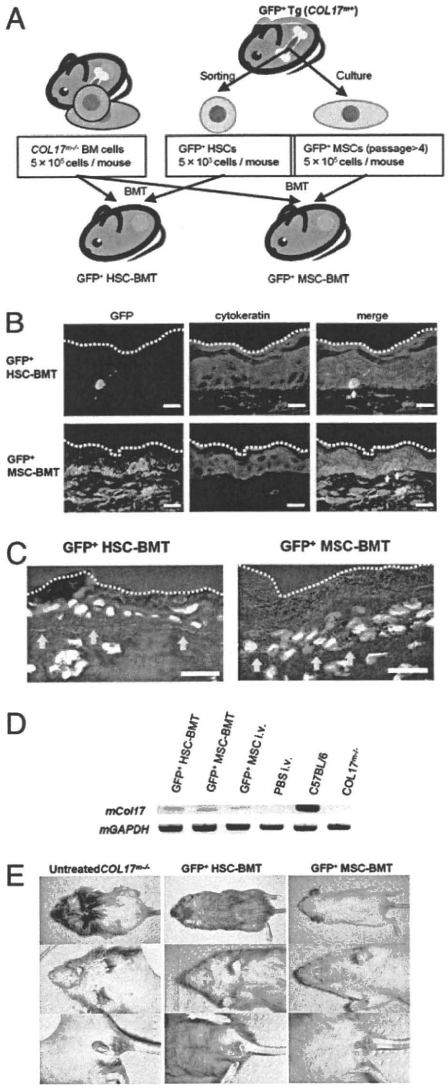

Fig. 4. HSCs and MSCs each have the potential to produce mCol17 in transplanted $COL17^{m-/-}$ mice. (*A*) HSCs and MSCs from GFP+ Tg mice were sorted or cultured. One or the other type of these stem cells with supporting $COL17^{m-/-}$ whole BM cells were injected into preirradiated $COL17^{m-/-}$ mice. (*B*) Sparse GFP+ cytokeratin+ cells, shown by white arrows, are detected in the epithelized skin of HSC-BMT model mouse (*Upper*). Also in the MSC-BMT model, GFP+ cytokeratin+ cells are observed (*Lower*). (Scale bars: 10 μm.) (*C*) Punctate staining of mCol17 is noted, shown as yellow arrows, in the epithelized skin tissue of both HSC- and MSC-BMT model mice. (Scale bars: 20 μm.) (*D*) RT-PCR analysis of the epithelized skin area after full-thickness wounding. Both HSCs (lane 1) and MSCs (lane 2) in the BMT treatment model express positive *mCol17*. Also, single i.v. injection of GFP+ MSCs (lane 3) induces weak *mCol17* mRNA expression. (*E*) At 90 d after treatment, the HSC-BMT model mice (*Center*) demonstrate better clinical manifestations than the untreated $COL17^{m-/-}$ mice (*Left*), whereas mice of the MSC-BMT models (*Right*) tend to show more severe perianal erosions and hair loss.

lamina and dermis. Recently, various treatments were reported to restore the deficient proteins. These approaches fall mainly into three strategies: gene therapy (20–24), protein therapy (21, 25, 26), and cell therapy. Cell therapies using fibroblasts have been attempted for recessive dystrophic EB (RDEB) model mice and human patients, both of which lack collagen VII. Intradermal fibroblast cell therapy was reported for RDEB model mice (18) and RDEB human patients (27). These approaches may prove to be fundamental treatments for EB. However, their effects are transient and occur only where the genes, proteins, or cells are introduced; they may cause rejection and such gene-correction approaches still raise questions of ethics and safety. Woodley et al. (28) recently reported that i.v. injection of human fibroblasts induces systemic production of human collagen VII in immunodeficient mice; however, major ethical and safety problems remain in human patients.

BMT, an established, widely used medical technique for hematologic malignancies, has recently been attempted for severe hereditary genetic disorders. Hobbs et al. (29) first reported the

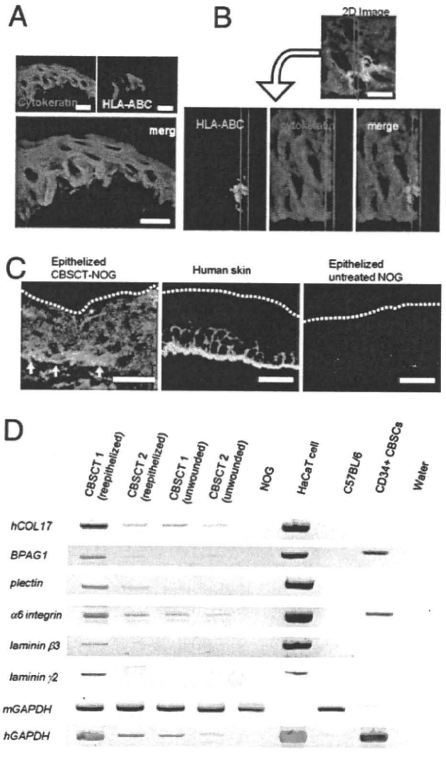

Fig. 5. Human hematopoietic stem cell transplantation induces human epidermal keratinocytes that produce BMZ proteins. (*A*) The epithelized skin samples of treated NOG mice include sporadic cytokeratin$^+$ (red), HLA-ABC$^+$ (blue) cells in the basal cell layer, which indicate human cord blood-derived keratinocytes. (Scale bars: 10 μm.) (*B*) 3D analyses of the immunohistochemical sections prove costaining of keratin (red) and HLA-ABC (green), indicating that these cells are human cord blood-derived keratinocytes and not two distinct overlaid cells. Blue lines: cross-section edges. (Scale bar: 10 μm.) (*C*) Sparse, linear deposition of hCOL17 is noted in the epithelized skin of CBSCT-treated NOG mice (yellow arrows). Green: hCOL17 (D20); red: cytokeratin; (Scale bars: 20 μm.) (*D*) RT-PCR analysis for two transplanted NOG mice, for both unwounded and epithelized skin (CBSCT 1 and CBSCT 2). The expression of several BMZ proteins, *hCOL17, BPAG1, plectin, α6 integrin, laminin β3*, and *laminin γ2* mRNA is demonstrated. Unwounded skin shows faint expression of *hCOL17* and *α6 integrin*.

efficacy of BMT for treatment of Hurler's syndrome. Later hematopoietic stem cell transplantation proved effective in other mucopolysaccharidoses (30–33). More recently, Sampaolesi et al. (34) reported on the potential of stem cell therapy for the treatment of Duchene muscular dystrophy. These experiments indicate that stem cell therapies including BMT are promising candidates for several congenital genetic disorders. BMT techniques have three advantages over previous cell therapies: (*i*) systemic and long-lasting effects can be expected from the circulating BM-derived cells, (*ii*) conventional BMT techniques can be used in clinical applications, and therefore (*iii*) fewer ethical problems arise from the treatment.

Recently Tolar et al. (35) reported that hematopoietic stem cells contributed to life prolongation in RDEB neonatal mice. Chino et al. (36) reported that treatment of embryonic BMT in RDEB mice induced the expression of donor-derived fibroblasts and type VII collagen. These reports investigated neonatal or embryonic mice and focused on type VII collagen, which is produced mainly by dermal fibroblasts. In this study we show the potential of BMT therapies in adult mice with cutaneous congenital disorders caused by deficiencies of transmembrane proteins such as COL17, which is produced mainly by keratinocytes.

As to the origin of the BM-derived cells that differentiated into epidermal cells, Tolar et al. reported that only CD150$^+$ CD48$^-$ HSCs contributed to the amelioration of RDEB mice; nevertheless, MSCs abundantly expressed type VII collagen mRNA (35). Conversely, we and other groups have reported that MSCs also have the potential to differentiate into keratinocytes (37, 38). Our experiments have shown that both HSCs and MSCs have the potential to produce Col17. However, a BMT model with infusion of enriched MSCs tended to induce more severe clinical manifestations. One hypothesis is that the infused MSCs in BMT reside so shortly that long-term effects, such as clinical improvement, might not occur (39, 40). We demonstrated that human cord blood CD34$^+$ HSCs are able to differentiate into keratinocytes in vivo. Although further investigation is needed, we suggest that the difference between the benefits of MSC and HSC transplantations owes to the lack of a long-term, renewable circulating source of Col17-producing cells induced by donor hematopoiesis. From these findings we conclude that both HSCs and MSCs contributed to the production of Col17, although HSCs played more a significant role in clinical improvement in our EB model mice.

Recently Kopp et al. (41) performed BMT and subsequent skin transplantation in one Herlitz-junctional EB infant but unfortunately the child died. They performed normal conditioning treatments similar to those used in hematological malignancies, but the treatments might be too strong for EB patients. Indeed, in our EB model mice, we had to reduce the irradiation dose before BMT to avoid erosions. This report is highly suggestive toward determining conditioning regimens.

In conclusion, we confirmed the reexpression of the previously deficient anchoring protein Col17, better clinical appearance, and longer life expectancy after BMT in our junctional EB model mice. Furthermore we demonstrated that human HSCs can contribute to the regeneration of wounded skin, producing structural proteins in the BMZ. Current conventional hematopoietic stem cell transplantation will lead to treatments for severe forms of EB or even other congenital skin disorders involving epidermal structural proteins.

Materials and Methods

Bone Marrow Transplantation. Recipient adult mice were irradiated with a lethal dose of X-rays at 9 Gy (C57BL/6 and *COL17*$^{m-/-,h+}$ mice) or 6 Gy (in *COL17*$^{m-/-}$ mice), 12 h before infusions. The 9-Gy irradiation resulted in severe erosions and hair loss within 4 wk after BMT to *COL17*$^{m-/-}$ mice. Approximately 3.0–6.0 × 10^6 murine BM-derived cells in 400 μL PBS were injected through the mouse tail vein.

Human Cord Blood Stem Cell Transplantation. Recipient adult NOG mice were irradiated with a sublethal dose of X-rays at 2.5 Gy. Twelve hours later, approximately 1.0–2.5 × 10^5 human CD34$^+$ cells in 400 μL PBS were injected through the mouse tail vein. Hematopoietic reconstitution was evaluated in peripheral blood mononuclear cells 12 wk after transplantation.

Hematopoietic and Mesenchymal Cell Transplantations. Recipient adult *COL17*$^{m-/-}$ mice were irradiated with a lethal dose of X-rays at 6 Gy. Twelve hours later, approximately 5.0 × 10^3 GFP$^+$ HSCs (n = 5) or 5.0 × 10^5 GFP$^+$ MSCs (n = 4) were mixed with 5.0 × 10^5 whole *COL17*$^{m-/-}$ BM-derived cells in 400 μL PBS and injected through the mouse tail vein.

RT-PCR Analyses. For RT-PCR, total RNA from tissues and cells was extracted using Isogen (Nippon Gene) and 200 ng of total RNA was used for cDNA synthesis in SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). RT-PCR analysis of mRNA was performed in a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer). The primers specific for protein sequences are summarized in Table S1. The PCR protocol for these genes included 35 cycles of amplification (denaturing at 94 °C for 1 min, annealing for 1 min, elongation at 72 °C for 1 min). Aliquots from each amplification reaction were analyzed by electrophoresis in 2% acrylamide-Tris-borate gels. Gel images were acquired and processed by an image analyzer (LAS-4000UVmini; Fujifilm).

Western Blotting. Protein lysates from epidermal tissues were subjected to SDS/PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with 1% nonfat dry milk in PBS, probed with rat monoclonal antibodies against mCol17 (KT4.2, 1:80,000), and then allowed to react with goat anti-rat IgG antibody coupled with HRP (1:1,000; Southern Biotech). For loading control, we used mouse anti-β-actin antibody

(1:1,000; Sigma-Aldrich) and HRP-conjugated goat anti-mouse IgG (1:1,000; Southern Biotech). The resultant immune complexes were visualized using a chemiluminescent detection system (LumiGLO; Cell Signaling Technology) and processed by an image analyzer (LAS-4000UVmini).

Ultrastructural Observations. Skin biopsy samples of two mice each from GFP$^+$ Tg mice, untreated *COL17*$^{m-/-}$ mice, and BMT-treated *COL17*$^{m-/-}$ mice were fixed in 5% glutaraldehyde solution, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. The samples were sectioned at 1-μm thickness for light microscopy and thin-sectioned at 70-nm thickness for electron microscopy. The thin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (H-7100; Hitachi High-Technologies).

Clinical Evaluation of *COL17*$^{m-/-}$ Mice. After bone marrow transplantation, the clinical severity of the BMT-treated *COL17*$^{m-/-}$ mice, such as spontaneous erosions and blistering, was evaluated and compared with that of the untreated *COL17*$^{m-/-}$ mice and BMT-control mice whose donors were *COL17*$^{m-/-}$ mice. The perioral area and circumanal area tend to be naturally predisposed to erosion. We investigated the clinical severity by measuring the share of each affected area as a percent of its entire region. These were assessed by two independent assessors viewing the same clinical images. In addition we compared the vital prognosis after BMT (35 d after birth) among BMT-treated mice (n = 20), untreated *COL17*$^{m-/-}$ mice (n = 17), and BMT-control mice (n = 6).

Mechanical Rubber Stress Test. The epithelized dorsal skin areas of 1 cm^2 after erosion were marked with a pen and exposed to a mechanical rubber stress test as previously reported (18). The skin was gently stretched, and mechanical shearing forces were applied by the same investigator repeatedly (25 times) as intense, unidirectional rubbing with a pencil eraser. After 20 min, skin specimens were excised and processed for histopathological analysis. Also, we measured the total areas of erosion by ImageJ software (42).

Statistical Analyses. Mann–Whitney U test for nonparametric data, Kaplan-Meier analysis for survival curves, and log-rank test for survival evaluation were performed using Excel 2003 (Microsoft) with the add-in software Statcel2 (OMS) (43). For comparison of more than two groups, data were analyzed by Kruskal–Wallis test followed by Scheffe's F test. Results were expressed as mean ± SE.

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1. Satake K, Lou J, Lenke LG (2004) Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue. *Spine (Phila Pa 1976)* 29:1971–1979.
2. Herzog EL, Chai L, Krause DS (2003) Plasticity of marrow-derived stem cells. *Blood* 102: 3483–3493.
3. Jiang Y, et al. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:41–49.
4. Orlic D, et al. (2001) Bone marrow cells regenerate infarcted myocardium. *Nature* 410: 701–705.
5. Kocher AA, et al. (2001) Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7:430–436.
6. Ferrari G, et al. (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528–1530.
7. Yamada M, et al. (2004) Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. *J Immunol* 172:1266–1272.
8. Kale S, et al. (2003) Bone marrow stem cells contribute to repair of the ischemically injured renal tubule. *J Clin Invest* 112:42–49.
9. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 297:2256–2259.
10. Lagasse E, et al. (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 6:1229–1234.
11. Fine JD, et al. (2008) The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. *J Am Acad Dermatol* 58:931–950.
12. Mavilio F, et al. (2006) Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat Med* 12:1397–1402.
13. Banasik MB, McCray PB, Jr (2010) Integrase-defective lentiviral vectors: Progress and applications. *Gene Ther* 17:150–157.
14. Murata H, et al. (2007) Donor-derived cells and human graft-versus-host disease of the skin. *Blood* 109:2663–2665.
15. Harris RG, et al. (2004) Lack of a fusion requirement for development of bone marrow-derived epithelia. *Science* 305:90–93.
16. Inokuma D, et al. (2006) CTACK/CCL27 accelerates skin regeneration via accumulation of bone marrow-derived keratinocytes. *Stem Cells* 24:2810–2816.
17. Nishie W, et al. (2007) Humanization of autoantigen. *Nat Med* 13:378–383.
18. Fritsch A, et al. (2008) A hypomorphic mouse model of dystrophic epidermolysis bullosa reveals mechanisms of disease and response to fibroblast therapy. *J Clin Invest* 118:1669–1679.
19. Ito M, et al. (2002) NOD/SCID/gamma(c)(null) mouse: An excellent recipient mouse model for engraftment of human cells. *Blood* 100:3175–3182.
20. Bauer JW, Lanschuetzer C (2003) Type XVII collagen gene mutations in junctional epidermolysis bullosa and prospects for gene therapy. *Clin Exp Dermatol* 28:53–60.
21. Robbins PB, Sheu SM, Goodnough JB, Khavari PA (2001) Impact of laminin 5 beta3 gene versus protein replacement on gene expression patterns in junctional epidermolysis bullosa. *Hum Gene Ther* 12:1443–1448.
22. Robbins PB, et al. (2001) In vivo restoration of laminin 5 beta 3 expression and function in junctional epidermolysis bullosa. *Proc Natl Acad Sci USA* 98:5193–5198.
23. Dellambra E, et al. (2001) Gene correction of integrin beta4-dependent pyloric atresia-junctional epidermolysis bullosa keratinocytes establishes a role for beta4 tyrosines 1422 and 1440 in hemidesmosome assembly. *J Biol Chem* 276:41336–41342.
24. Seitz CS, Giudice GJ, Balding SD, Marinkovich MP, Khavari PA (1999) BP180 gene delivery in junctional epidermolysis bullosa. *Gene Ther* 6:42–47.
25. Igoucheva O, Kelly A, Uitto J, Alexeev V (2008) Protein therapeutics for junctional epidermolysis bullosa: Incorporation of recombinant beta3 chain into laminin 332 in beta3-/- keratinocytes in vitro. *J Invest Dermatol* 128:1476–1486.
26. Woodley DT, et al. (2004) Injection of recombinant human type VII collagen restores collagen function in dystrophic epidermolysis bullosa. *Nat Med* 10:693–695.
27. Wong T, et al. (2008) Potential of fibroblast cell therapy for recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 128:2179–2189.
28. Woodley DT, et al. (2007) Intravenously injected human fibroblasts home to skin wounds, deliver type VII collagen, and promote wound healing. *Mol Ther* 15:628–635.
29. Hobbs JR, et al. (1981) Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone-marrow transplantation. *Lancet* 2:709–712.
30. Sands MS, et al. (1997) Murine mucopolysaccharidosis type VII: Long term therapeutic effects of enzyme replacement and enzyme replacement followed by bone marrow transplantation. *J Clin Invest* 99:1596–1605.
31. Warkentin PI, Dixon MS, Jr, Schafer I, Strandjord SE, Coccia PF (1986) Bone marrow transplantation in Hunter syndrome: A preliminary report. *Birth Defects Orig Artic Ser* 22:31–39.
32. Krivit W, et al. (1984) Bone-marrow transplantation in the Maroteaux-Lamy syndrome (mucopolysaccharidosis type VI). Biochemical and clinical status 24 months after transplantation. *N Engl J Med* 311:1606–1611.
33. Gasper PW, et al. (1984) Correction of feline arylsulphatase B deficiency (mucopolysaccharidosis VI) by bone marrow transplantation. *Nature* 312:467–469.
34. Sampaolesi M, et al. (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* 444:574–579.
35. Tolar J, et al. (2009) Amelioration of epidermolysis bullosa by transfer of wild-type bone marrow cells. *Blood* 113:1167–1174.
36. Chino T, et al. (2008) Bone marrow cell transfer into fetal circulation can ameliorate genetic skin diseases by providing fibroblasts to the skin and inducing immune tolerance. *Am J Pathol* 173:803–814.
37. Wu Y, Chen L, Scott PG, Tredget EE (2007) Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 25:2648–2659.
38. Sasaki M, et al. (2008) Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol* 180:2581–2587.
39. Rieger K, et al. (2005) Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation. *Exp Hematol* 33:605–611.
40. Dickhut A, et al. (2005) Mesenchymal stem cells obtained after bone marrow transplantation or peripheral blood stem cell transplantation originate from host tissue. *Ann Hematol* 84:722–727.
41. Kopp J, et al. (2005) Hematopoietic stem cell transplantation and subsequent 80% skin exchange by grafts from the same donor in a patient with Herlitz disease. *Transplantation* 79:255–256.
42. Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image Processing with ImageJ. *Biophotonics International* 11:36–42.
43. Yanai H (2004) *Statcel-The Useful Add-In Software Forms on Excel* (OMS, Tokyo) 2nd Ed.

Corrections and Retraction

CORRECTIONS

GENETICS

Correction for "Lack of association of common variants on chromosome 2p with primary open-angle glaucoma in the Japanese population," by Fumihiko Mabuchi, Yoichi Sakurada, Kenji Kashiwagi, Zentaro Yamagata, Hiroyuki lijima, and Shigeo Tsukahara, which appeared in issue 21, May 25, 2010, of *Proc Natl Acad Sci USA* (107:E90–E91; first published April 27, 2010; 10.1073/pnas.0914903107).

The authors note that, due to a printer's error, the author name Hiroyuki lijima should have appeared as Hiroyuki Iijima. The corrected author line appears below. The online version has been corrected.

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MEDICAL SCIENCES

Correction for "Bone marrow transplantation restores epidermal basement membrane protein expression and rescues epidermolysis bullosa model mice," by Yasuyuki Fujita, Riichiro Abe, Daisuke Inokuma, Mikako Sasaki, Daichi Hoshina, Ken Natsuga, Wataru Nishie, James R. McMillan, Hideki Nakamura, Tadamichi Shimizu, Masashi Akiyama, Daisuke Sawamura, and Hiroshi Shimizu, which appeared in issue 32, August 10, 2010, of *Proc Natl Acad Sci USA* (107:14345–14350; first published July 26, 2010; 10.1073/pnas.1000044107).

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RETRACTION

MEDICAL SCIENCES

Retraction for "Complete and persistent phenotypic correction of phenylketonuria in mice by site-specific genome integration of murine phenylalanine hydroxylase cDNA," by Li Chen and Savio L. C. Woo, which appeared in issue 43, October 25, 2005, of *Proc Natl Acad Sci USA* (102:15581–15586; first published October 17, 2005; 10.1073/pnas.0503877102).

The undersigned author wishes to note the following: "After re-examining the laboratory records, I have concluded that there are data irregularities underlying this paper that warrant its retraction. I regret not recognizing these irregularities before the manuscript was published and apologize for any inconvenience this might have caused."

Savio L. C. Woo

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Topical application of anti-angiogenic peptides based on pigment epithelium-derived factor can improve psoriasis

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ABSTRACT

Background: Psoriasis is a common chronic inflammatory skin disorder with a high prevalence (3–5%) in the Caucasian population. Although the number of capillary vessels increases in psoriatic lesions, there have been few reports that have specifically examined the role of angiogenesis in psoriasis. Angiogenic factors, such as vascular endothelial growth factor (VEGF), may dominate the activity of anti-angiogenic factors and accelerate angiogenesis in psoriatic skin.
Objective: We investigated to identify small peptide mimetics of PEDF that might show anti-angiogenic potential for the topical treatment for psoriasis.
Methods: We examined the expression of PEDF in skin by immunohistochemical staining, immunoblotting, and RT-PCR. To identify potential PEDF peptides, we screened peptides derived from the proteolytic fragmentation of PEDF for their anti-proliferative action. Anti-psoriatic functions of these peptides were analyzed using a mouse graft model of psoriasis.
Results: The specific low-molecular weight peptides (MW < 850 Da) penetrated the skin and showed significant anti-angiogenic activity in vitro. Topical application of these peptides in a severe combined immunodeficient mouse model of psoriatic disease led to reduced angiogenesis and epidermal thickness.
Conclusions: These data suggest that low-molecular PEDF peptides with anti-angiogenic activity may be a novel therapeutic strategy for psoriasis.

1. Introduction

Psoriasis is a common skin disease affecting 0.5–3% of the Caucasian population [1]. Histopathologically, this disorder is characterized by accelerated epidermal proliferation, by the infiltration of inflammatory cells into the epidermis and upper dermis, and by telangiectasia in the superficial dermis. Although the molecular pathogenesis of psoriasis remains unclear, several hypotheses have been proposed. Activated T lymphocytes infiltrate into the lesional skin areas where they secrete a variety of cytokines such as tumor necrosis factor (TNF)-α, interferon-γ, IL-2 and IL-12, and thus play an important role in psoriatic inflammatory changes [2]. In addition, epidermal proliferation is influenced by inappropriate vascular expansion in the superficial dermis [3]. Furthermore, these microvascular changes in psoriatic skin lesions include pronounced capillary dilatation, increased vessel permeability and endothelial cell proliferation and protrusion into the dermal papillae capillaries. Therefore inappropriate angiogenic growth has been proposed to contribute to the pathogenesis of psoriasis [4,5]. The overexpression of angiogenic factors also occurs; for instance, vascular endothelial growth factor (VEGF) is strongly up-regulated in psoriatic skin lesions [6].

There have been a number of therapeutic strategies devised for psoriasis. Topical steroids, topical vitamin D3 analogs, oral retinoids, UV irradiation such as PUVA and narrow-band UVB, cyclosporine and other immunosuppressants have been widely used. In addition, biological agents that target cytokines such as TNF-α have recently been developed [7]. Most strategies are aimed at reducing the inflammatory reaction and epidermal proliferation, but yet there have been few agents targeting angiogenesis in psoriasis.

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors, and it was

Abbreviations: PEDF, pigmented epithelium-derived factor; VEGF, vascular endothelial growth factor.

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