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Fibroblasts Show More Potential as Target Cells than Keratinocytes in *COL7A1* Gene Therapy of Dystrophic Epidermolysis Bullosa

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Dystrophic epidermolysis bullosa (DEB) is an inherited blistering skin disorder caused by mutations in the type VII collagen gene (*COL7A1*). Therapeutic introduction of *COL7A1* into skin cells holds significant promise for the treatment of DEB. The purpose of this study was to establish an efficient retroviral transfer method for *COL7A1* into DEB epidermal keratinocytes and dermal fibroblasts, and to determine which gene-transferred cells can most efficiently express collagen VII in the skin. We demonstrated that gene transfer using a combination of G protein of vesicular stomatitis virus-pseudotyped retroviral vector and retronectin introduced *COL7A1* into keratinocytes and fibroblasts from a DEB patient with the lack of *COL7A1* expression. Real-time polymerase chain reaction analysis of the normal human skin demonstrated that the quantity of *COL7A1* expression in the epidermis was significantly higher than that in the dermis. Subsequently, we have produced skin grafts with the gene-transferred or untreated DEB keratinocytes and fibroblasts, and have transplanted them into nude rats. Interestingly, the series of skin graft experiments showed that the gene-transferred fibroblasts supplied higher amount of collagen VII to the new dermal–epidermal junction than the gene-transferred keratinocytes. An ultrastructural study revealed that collagen VII from gene-transferred cells formed proper anchoring fibrils. These results suggest that fibroblasts may be a better gene therapy target of DEB treatment than keratinocytes.

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INTRODUCTION

Type VII collagen, a non-fibrillar collagen, is a major component of anchoring fibril loop structures beneath the epidermal basement membrane (Uitto *et al.*, 1992; Burgeson, 1993). Cloning of collagen VII cDNA demonstrated a primary sequence of 2,944 amino acids and the basic organization of the functional domains (Christiano *et al.*, 1994a). Subsequent genomic cloning has highlighted the structural organization of the collagen VII gene (*COL7A1*) (Christiano *et al.*, 1994b). This cloning information has enabled genomic DNA sequence analysis of *COL7A1* and has demonstrated that mutations within *COL7A1* are associated with the dystrophic forms of epidermolysis bullosa (DEB). DEB comprises a group of mechanobullous diseases characterized by cutaneous fragility with a tendency to form sub-basal lamina densa blisters (Christiano *et al.*, 1993; Pulkkinen and Uitto, 1999; Chen *et al.*, 2002a). In addition, targeted disruption of *COL7A1* in a mouse model demonstrated an almost identical phenotype to DEB in humans (Heinonen *et al.*, 1999). These results indicate that collagen VII is of critical importance for dermal–epidermal adhesion.

Approximately 300 distinct *COL7A1* mutations have been identified in DEB patients so far. Therapeutic introduction of *COL7A1* into skin cells is a promising treatment of DEB. Despite the relatively large size of *COL7A1*, the cDNA of which is still 9 kb makes gene transfer relatively problematic, and several methods including lentivirus- (Chen *et al.*, 2002b), retrovirus- (Baldeschi *et al.*, 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al.*, 2002) have attempted to transfer *COL7A1* into keratinocytes. These studies used keratinocytes as target cells as collagen VII has been reported to be mainly synthesized and secreted by keratinocytes and to lesser extent by fibroblasts (Ryynanen *et al.*, 1992). However, application of gene-transferred DEB fibroblasts into the skin restored collagen VII expression in the dermal–epidermal junction (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003). In addition, using an intradermal injection of lentivirus with *COL7A1* induced the expression of collagen VII in fibroblasts and endothelial cells, resulting in collagen VII accumulation in the grafted DEB skin on the host animal (Woodley *et al.*, 2004).

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Abbreviations: DEB, dystrophic epidermolysis bullosa; FCS, fetal calf serum; VSV-G, G protein of vesicular stomatitis virus

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In this study, we have established a retroviral method to transfer *COL7A1* into DEB keratinocytes and fibroblasts. Next, we produced the skin grafts with gene-transferred keratinocytes or fibroblasts, and transplanted them into nude rats. Examination of collagen VII graft expression revealed that gene-transferred fibroblasts assembled more collagen VII in the form of anchoring fibrils beneath the basement membrane than gene-transferred keratinocytes. We conclude that fibroblasts are a more ideally suited target for *COL7A1* gene transfer than keratinocytes using retroviral gene therapy for the treatment of DEB.

RESULTS

Successful transfer COL7A1 using retroviral systems

We employed two retroviral vectors, pLIXN and pDON-AI, and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids termed pLI-COL and pDON-COL, respectively (Figure 1). Also, we created pDON(Δ) by removing the SV-40 promoter and *Neo* gene from pDON-AI and constructed a retroviral vector with *COL7A1* cDNA pDON(Δ)-COL (Figure 1). Several series of preliminary experiments demonstrated that retronectin (TAKARA) increased attachment of virus to keratinocytes and fibroblasts. Also, use of plasmid G protein of vesicular stomatitis virus (pVSV-G) (Pantropic System; Clontech, Palo Alto, CA) enabled concentration of viral particles by ultracentrifugation, resulting in an increase of transfer efficacy. After transfection of plasmids pLI-COL, pDON-COL, and pDON(Δ)-COL to 293 packaging cells, the culture media were collected. The virus titers (mean $\pm$ SD $\times 10^6$/ml) of pLI-COL, pDON-COL, and pDON(Δ)-COL were $1.1 + 0.35$, $1.6 + 0.46$, and $2.7 + 0.55$, respectively.

We transferred *COL7A1* into cultured DEB keratinocytes and fibroblasts using the retroviral system. Transfection experiments showed that retroviral methods using pLI-COL and pDON-COL failed to introduce *COL7A1* to DEB cells (data not shown). Plasmid pDON(Δ)-COL with the VSV-G system allowed a greater gene transfer after further concentration of the virus particles increased the transfer rate (Figure 2). Immunostaining revealed that the transfer rates in DEB keratinocytes and fibroblasts were almost the same (Figures 2 and 3a), and immunoblotting demonstrated that the amount of collagen VII in their culture media was also identical (Figure 3b). The average copy number per cell of the *COL7A1* cDNA was evaluated by Southern blot analysis of genomic DNA extracted from the transduced cells. The result indicated that the intensities of 7.2 kb band from the integrated cDNA were the same in treated keratinocytes and fibroblasts, suggesting that integration copies for keratinocytes and fibroblasts was almost equal (Figure 3c). The copy number was estimated 2–3 by comparing with a serial dilution standard (data not shown).

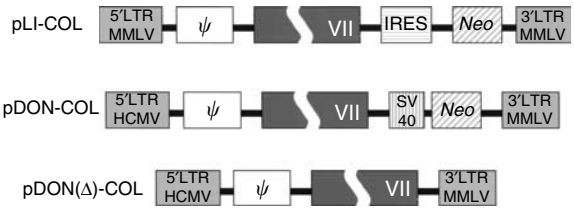

Figure 1. Schematic representation of retroviral *COL7A1* expression vectors. We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (Takara, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids pLI-COL and pDON-COL, respectively. A pDON(Δ) vector was created by removing the SV-40 promoter and *Neo* gene from pDON-AI and *COL7A1* cDNA constructed retroviral vector made termed pDON(Δ)-COL. These vectors harbor long terminal repeat (LTR) derived from mouse moloney leukemia virus (MMLV) and human cytomegalovirus (HCMV). The internal ribosome entry site (IRES) enables expression of two unrelated reading frames from a single transcription unit. ψ: packaging signal.

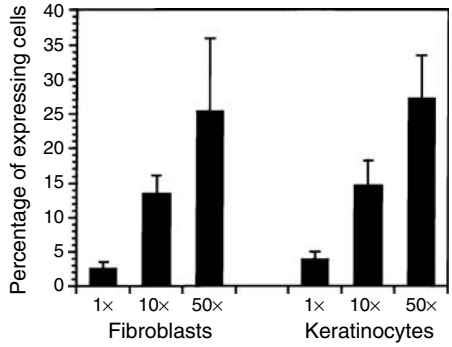

Figure 2. Successful gene transfer of *COL7A1* into DEB fibroblasts and keratinocytes using retroviral systems. *COL7A1* was transferred into DEB fibroblasts and keratinocytes using the retroviral systems. Immunostaining revealed that the transfer rates in DEB keratinocytes and fibroblasts were almost equal. The concentration of virus particles (by 10 or 50 times) using the VSV-G system improves the transfer rate. The values were represented the mean $\pm$ SD of six individual samples.

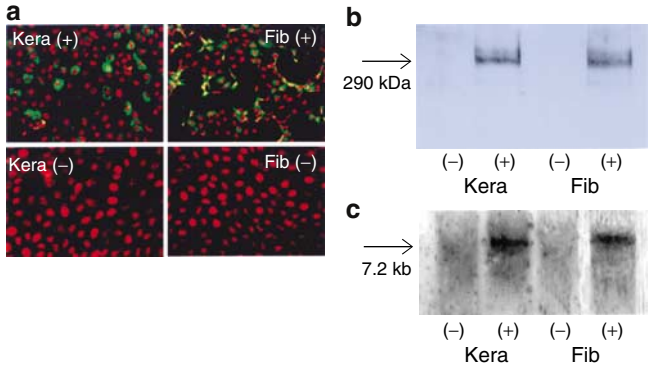

Figure 3. Corrective gene transfer of the *COL7A1* into DEB fibroblasts and keratinocytes. (**a**) Immnostaining showed that the gene-transfected DEB keratinocytes Kera (+) and fibroblasts Fib (+) expressed collagen VII, whereas no expression was found in either the untreated keratinocytes Kera (–) or fibroblasts Fib (–). Nuclei were counterstained with propidium iodide. (**b**) Western blot analysis demonstrated that the amount of transgene product in culture medium was almost the same between keratinocytes and fibroblasts. (**c**) Southern blot analysis of genomic DNA extracted from the transduced cells showed that the *COL7A1* cDNA integration copies for keratinocytes and fibroblasts were almost equal.

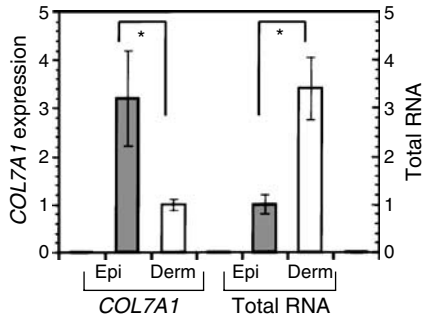

Figure 4. *In vivo COL7A1* expression in the epidermis is higher than that in the dermis. We measured *COL7A1* mRNA levels in the epidermis (Epi) and dermis (Derm) *in vivo*. Real-time PCR demonstrated that the *COL7A1*-specific signal (per RNA) of the epidermis was higher than that of the dermis. Comparison of the total RNA amounts from the epidermis and dermis in the same area of the excised normal skin showed that the amount from the dermis was higher than that from the epidermis. The *COL7A1* mRNA expression and total RNA amounts were expressed as an arbitrary scale. The values were represented the mean ± SD from three separate samples. $*P<0.01$, significant difference.

In vivo COL7A1 expression in epidermis is higher than that in dermis

To determine the *COL7A1* expression level in the epidermis and dermis *in vivo,* we separated the epidermis from the dermis, and measured *COL7A1* mRNA levels using real-time PCR. Real-time PCR demonstrated that the *COL7A1*-specific signal (per RNA) of the epidermis was higher than that of the dermis by 3.2-fold (Figure 4). Comparison of the total RNA amounts from the epidermis and dermis in the same area of the excised normal skin showed that the amount from the dermis was higher than that from the epidermis by 3.4-fold (Figure 4). Thus, the quantity of *COL7A1* expression in epidermis was significantly higher than that in dermis *in vivo*.

Gene-transferred fibroblasts can supply more collagen VII to the basement membrane zone than gene-transferred keratinocytes

We transplanted the gene-transferred DEB keratinocytes and fibroblasts into the wound of nude rats, and then observed *COL7A1* deposition 3, 6, and 9 weeks after transplantation. In the skin graft with gene-transferred keratinocytes and untreated fibroblasts, the *COL7A1* deposition was detectable in the basement membrane zone at 3 week and maintained this expression at least until 9 weeks (Figure 5). However, we found a greater accumulation of collagen VII in dermal–epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts 3 weeks after transplantation. Furthermore, DEB fibroblasts transfected with *COL7A1* demonstrated more dermal–epidermal junction collagen VII staining than *COL7A1*-transfected DEB keratinocytes/untreated fibroblast (Figure 5) from 6 to 9 weeks. The grafts of DEB keratinocytes and fibroblasts as controls demonstrated no deposition (Figure 5). Semiquantification of *COL7A1* deposition in basement membrane zone in each point showed DEB fibroblasts with COL7A1 can supply higher amount of collagen VII to the basement membrane zone than DEB keratinocytes with *COL7A1* (Figure 6). Significant differences were found between the keratinocytes and fibroblasts samples at 3W and 9W points.

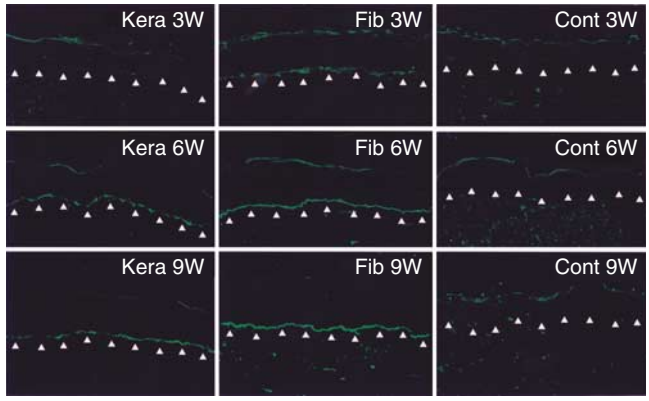

Figure 5. Gene-transferred fibroblasts can supply more collagen VII to the sub-basement membrane zone than gene-transferred keratinocytes. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6, and 9 weeks after transplantation. The skin graft with gene-transferred keratinocytes and untreated fibroblasts (Kera 3W, Kera 6W, Kera 9W) started dermal–epidermal junction collagen VII deposition at 3 week and maintained it until 9 weeks. A greater accumulation of collagen VII in dermal–epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts was found 3 weeks after transplantation (Fib 3W). The DEB fibroblasts transfected with *COL7A1* demonstrated more dermal–epidermal junction collagen VII staining than *COL7A1*-transfected DEB keratinocytes/untreated fibroblast from 6 to 9 weeks (Fib 6W, Fib 9W). The control DEB keratinocyte and fibroblast cell grafts (Cont 3W, Cont 6W, Cont 9W) demonstrated no deposition. Arrowheads define the limit between the dermis and epidermis.

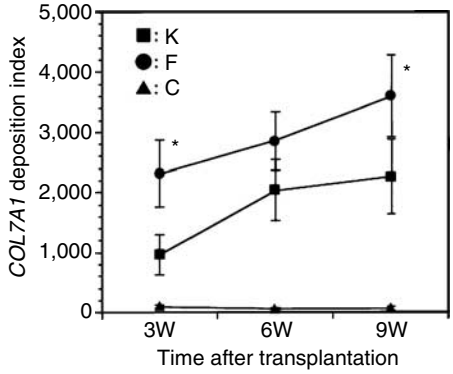

Figure 6. Semiquantification of *COL7A1* deposition in basement membrane zone. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6, and 9 weeks after transplantation. To semiquantify *COL7A1* deposition in basement membrane zone, we measured fluorescence intensity (arbitrary scale) in basement membrane zone at 10 areas at each point and the *COL7A1* deposition index was expressed as the mean ± SD from the 10 values. K: the skin graft with gene-transferred keratinocytes and untreated fibroblasts; F: the graft with untreated keratinocytes and gene-transferred fibroblasts grafts; C: the graft with untreated keratinocyte and untreated fibroblasts. $*P<0.01$, significant difference.

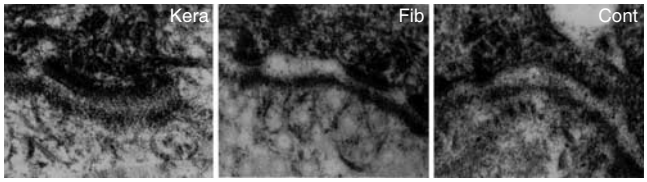

Figure 7. Collagen VII released from gene-transferred cells forms anchoring fibrils similar to normal skin. We examined the ultrastructural formation of anchoring fibrils in the grafts. The grafts with the gene-transferred keratinocytes (Kera) or the gene-transferred fibroblasts (Fib) demonstrated cross-banded, filamentous structures sometimes forming semicircular loops immediately beneath the lamina densa, corresponding to anchoring fibrils, whereas we failed to identify these filamentous structures in control without *COL7A1* transfection (Cont).

Collagen VII released from gene-transferred cells forms ultrastructurally normal anchoring fibrils

We examined the ultrastructural formation of anchoring fibrils in the graft. The grafts with both gene-transferred keratinocytes and fibroblasts demonstrated filamentous loop structures just beneath the lamina densa, which were corresponding to anchoring fibrils (Figure 7). We could not see any filamentous structures in control (untransfected) samples without *COL7A1*.

DISCUSSION

The developments in cloning the basement membrane protein genes have allowed the identification of the causative genes/proteins harboring the mutations responsible for this group of epidermolysis bullosa diseases (Uitto and Pulkkinen, 2001). We can now make good estimations about the prognosis and severity of these diseases with profound beneficial effects on genetic counseling and DNA-based prenatal diagnosis. However, patients most frequently desire an appropriate therapy for epidermolysis bullosa. Corrective transfer of the *COL7A1* gene back into the skin cells is a promising treatment of DEB.

The previous Northern hybridization study revealed a high level of *COL7A1* mRNA expression in cultured epidermal keratinocytes, whereas the expression was lower in cultured dermal fibroblasts (Ryynanen *et al.*, 1992). These results indicate that epidermal keratinocytes and dermal fibroblasts express the collagen VII, but also suggest that epidermal keratinocytes are the primary source of collagen VII in developing human skin. Many investigators have utilized keratinocytes as the target cells of DEB gene therapy. Several methods including viral- (Ghazizadeh and Taichman, 2000) and non-viral-mediated transduction (Vogel, 2000) have been reported for *in vivo* and *ex vivo* gene transfer into keratinocytes. *COL7A1* cDNA was recently transferred into cultured DEB keratinocytes using some methods including lentivirus- (Chen *et al.*, 2002b), retrovirus- (Baldeschi *et al.*, 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al.*, 2002). The corrected DEB keratinocytes expressed the recombinant collagen VII and restored the *in vivo* synthesis of anchoring fibrils after implantation, demonstrating the feasibility of gene transfer using DEB keratinocytes. We also succeeded in transferring the *COL7A1* into *in vivo* keratinocytes using the naked DNA method (Sawamura *et al.*, 2002) although the *COL7A1* transfer efficacy was lower than the above *ex vivo* method.

Many gene therapy protocols have already utilized retroviral vectors for clinical practices. In this study, we also showed a retroviral vector could transfer the 9 kb *COL7A1* cDNA into DEB keratinocytes. Another group has succeeded in transducing *COL7A1* gene to keratinocytes using a retroviral plasmid containing the Neo selection gene (Baldeschi *et al.*, 2003). They showed that transduction efficacies to primary keratinocytes were 40 and 83–93% by retroviral vectors pLSRS-Ires-zero and pMSCV, respectively. However, the use of similar plasmids is not possible to efficiently introduce this gene into keratinocytes in our experiments. In our system, the efficacy was about 30%, which was lower than those in previous report. This study, as far as we know, has been the first to try a retronectin retroviral targeting system for keratinocytes. Retronectin is a recombinant peptide, which consists of three functional fibronectin domains and significantly enhances retrovirus-mediated gene transduction into mammalian cells. Our data showed that addition of retronectin increased transfer efficacy in keratinocytes by 3-fold (data not shown), indicating that retronectin is indeed efficient in this keratinocyte/retroviral system.

Some groups have succeeded in transferring this gene into keratinocytes as mentioned above. On the other hand, cutaneous injection of the DEB fibroblasts transduced using ϕC31 integrase-based approach also restores collagen VII deposition along the dermal–epidermal junction (Ortiz-Urda *et al.*, 2003). Also, gene-corrected DEB fibroblasts and normal human fibroblasts alone could supply type VII collagen deposition at the basement membrane zone *in vivo* (Woodley *et al.* 2003), and this implies a possibility that normal cultured human dermal fibroblasts are injected intradermally into recesive dystrophic epidermolysis bullosa patients' skin. Moreover, intradermal injection of lentiviral vector with *COL7A1* increased collagen VII expression in fibroblasts and endothelial cells, resulting in stronger deposition of collagen VII along the basement membrane zone anchoring fibrils as seen by electron microscopy (Woodley *et al.*, 2004). This study also introduced the *COL7A1* gene into DEB fibroblasts using the retroviral method and the consequent collagen VII assembly beneath the basement membrane of the fibroblast containing graft.

In this study, we compared dermal fibroblasts and epidermal keratinocytes as efficient target recipient cells for the collagen VII transgene product. After retroviral introduction of *COL7A1*, the transfer efficacy and the amount of collagen VII in the cultured keratinocytes media supernatant are almost the same as those of fibroblasts. Interestingly, a series of skin graft experiments first demonstrated that gene-transfected fibroblasts more efficiently assembled collagen VII into the dermal–epidermal junction than the gene-transferred keratinocytes. Previous Northern blotting analysis revealed higher level of *COL7A1* mRNA expression in cultured epidermal keratinocytes than fibroblasts (Ryynanen

et al., 1992). This study utilized real-time PCR technique and confirmed that the epidermis produced much more collagen VII than the dermis *in vivo*. If gene-transferred fibroblasts and keratinocytes express similar amounts of type VII collagen also *in vivo*, the fibroblasts may have a better ability to supply type VII collagen to the basement membrane than the keratinocytes.

It is evident that expression of recombinant collagen VII is driven by heterologous promoters, which escape the regulatory mechanisms that govern the expression of endogenous collagen VII in the different cell types. Also, keratinocytes have been preferred because of the possibility they offer of targeting stem cells and compared to keratinocytes, fibroblasts rapidly senesce *in vivo* (Krueger, 2000). However, fibroblasts are more robust and less susceptible to growth arrest and differentiation than epidermal keratinocytes (Ortiz-Urda *et al.*, 2003). Furthermore, genetically engineered fibroblasts have had their use explored for therapeutic applications including visceral and cutaneous implantation to supply gene products to circulation (Roth *et al.*, 2001). Given the above combined factors, it is proposed that fibroblasts may be potentially more feasible and a better target of DEB gene therapy than keratinocytes.

MATERIALS AND METHODS

Cell culture

Primary keratinocytes were isolated and grown in the presence of an irradiated 3T3 feeder layer (Rheinwald and Green, 1975). Briefly, keratinocytes, which were obtained from skin biopsy of a DEB patient and healthy controls, were cultured on feeder layers of mitomycin C-treated mouse 3T3 fibroblasts in DMEM: Ham's F-12 (3:1) supplemented with 10% fetal calf serum (FCS), 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, and 8 ng/ml cholera toxin. The DEB patient was diagnosed as the most severe subtype, and Hallopeau–Siemens type showed no *COL7A1* expression in the skin and harbored heterozygous premature stop codon mutations 1474del8 and 5818delC. Fibroblasts were also obtained from skin biopsy from the DEB patient and healthy controls, and were cultured in DMEM with 10% FCS. Packaging cells amphopack-293 and GP2-293 (Clontech, Palo Alto, CA) were maintained in DMEM with 10% FCS, 2 mM glutamine, and 2 mM sodium pyruvate.

Informed consents were obtained from all individual subjects in this study. The protocols were approved by the Ethical Committee at Hokkaido University Graduate School of Medicine. This study was conducted according to the Declaration of Helsinki Principles.

Intrinsic expression of collagen VII in control keratinocytes and fibroblasts

Human skin was obtained from normal volunteers, and treated with 10 mg/ml dispase for 3 hours at 37°C to separate the epidermis from the dermis. The epidermal and dermal sheets were minced and total RNA was extracted using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with reverse transcriptase (Life Sciences Inc., St Petersburg, FL) using an oligo-dT primer. Assays-on-Demand™ Products for *COL7A1* and *GAPDH* were purchased from Applied Biosystems (Foster City, CA). The 50 μl reaction in each well contained 1 μl of total cDNA, 300 nM sequence-specific primers, and 200 nM dual-labeled fluorogenic probe with 1 U of Taqman Universal PCR master mix (Applied Biosystems). A negative control PCR without template and a positive PCR control with a template of known amplification were included in each assay. The samples underwent the following stages: stage 1, 50°C for 2 minutes; stage 2, 95°C for 10 minutes; and stage 3, 95°C for 15 seconds, followed by 60°C for 1 minutes. Stage 3 was repeated 45 times. Gene-specific products were measured by means of an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA) continuously for 45 cycles. The *COL7A1*-specific signal was normalized by constitutively expressed *GAPDH* and expressed as arbitrary scale.

Construction of retroviral COL7A1 expression vectors and transfection

Human full-length *COL7A1* cDNA was constructed from several overlapping cDNA clones (Sawamura *et al.*, 2002). We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (Takara, Otsu, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids termed pLI-COL and pDON-COL, respectively (Figure 1). Also, we created pDON(Δ) by removing the SV-40 promoter and *Neo* gene from pDON-AI and constructed a retroviral vector with *COL7A1* cDNA pDON(Δ)-COL (Figure 1). The recombinant retroviruses were produced by transfecting the retroviral plasmids into the amphotropic amphopack-293 packaging cells (Clontech) using calcium-phosphate coprecipitation. In addition, we tried VSV-G-pseudotyped retrovirus vectors. The retroviral plasmids and plasmid pVSV-G were cotransfected into pantropic GP2-293 packaging cells (Clontech). The viral particles were recovered from the cell culture medium 48 hours later and applied to keratinocyte or fibroblast cultures. To increase transfer efficacy, ultracentrifugation was performed to concentrate the VSV-G virus particles. The titer of the viral supernatant was determined by real-time quantitative PCR (Towers *et al.*, 1999).

Cells infection with retrovirus

Keratinocytes and fibroblasts were cultured to up to 60% of confluency and then infected with the viral suspensions in 5 μg/ml polybrene. To increase the virus–cell interactions, we coated the surface of the culture plates with 10 ng/ml retronectin (Takara; fibronectin fragment CH-296). After incubation for 24 hours at 32°C, we maintained the treated cells under fresh medium for another 24 hours until the transduction efficiency was assessed by immunofluorescence examination of the infected cells.

Immunostaining and immunoblot

Transfected cultured keratinocytes and fibroblasts were fixed with 2% parafromaldehye in phosphate-buffered saline, and were then incubated with the monoclonal antibody LH7.2 (1:100) against the NC1 domain of collagen VII (Chemicon, Temecula, CA) for 18 hours at 4°C. They were treated with secondary goat anti-mouse IgG antibodies conjugated with FITC (1:50) for 1 hour at 37°C, and preparations were examined under a fluorescence microscope. Nuclei were counterstained with propidium iodide (Dojindo Laboratories, Kumamoto, Japan). Subconfluent cell cultures were fed for 48 hours with serum-free medium supplemented with 50 μg/ml ascorbic acid. For SDS-PAGE analysis, the culture medium was

treated with Amicon Ultra-100,000 Centrifugal Filter Devices (Millipore, Bedford, MA) for concentration and desalting. The samples were separated on a 5% polyacrylamide gel under reducing conditions. Immunoblotting analysis was performed by treating with the LH7.2 monoclonal antibody (1:1,000) for 18 hours at 4°C, and then secondary goat anti-mouse IgG antibodies conjugated with peroxidase (1:2,000) for 1 hour at 37°C. The resultant complexes were processed for Phototope HRP Western Blot Detection System (Cell Signaling, Beverly, MA) according to the manufacturer's protocol.

Southern blot analysis

The average copy number per cell of the *COL7A1* cDNA was evaluated by Southern blot analysis (Baldeschi *et al.*, 2003). Briefly, genomic DNA was extracted from subconfluent cell cultures and digested with *Bgl*II and *Hind*III. Plasmid pDON(Δ)-COL was serially diluted with yeast genomic DNA at the final concentration ranging from 0.5 to 20 copies/cell. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA). The 375 bp cDNA extending from exons 58 to 64 was generated by PCR amplification of *COL7A1* cDNA as a template. This fragment was designed to recognize a 7.2 kb band from the integrated cDNA, and also 1.0 and 0.3 kb bands from the intrinsic *COL7A1* gene. The membranes were hybridized with the 375 bp cDNA probe was labeled by random primed incorporation of digoxigenin-labeled 2′-deoxyuridine 5′-triphosphate using the DIG DNA Labeling Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. After high stringency washes, blots were visualized using an enhanced chemiluminescence system.

Grafting of gene-transferred DEB cells

Gene-transferred and untreated DEB keratinocytes and DEB fibroblasts were cultured using the above methods. Fibroblasts (10^6) were seeded into a collagen sponge scaffold and maintained in DMEM with 10% FCS for 3 days. In nude rats (F344/N Jcl-rnu), the sites for transplantation were prepared by excising a 2 cm^2 area of dorsal epidermis and dermis, and then the collagen sponge (3 cm^2) containing the fibroblasts was placed into the skin wound. The confluent cultures of 10^6 keratinocytes were treated with dispase (1 nU/ml; Godo Shusei, Tokyo, Japan), and the floating epidermal sheet placed on the collagen sponge. Preliminary experiment showed that the number of fibroblasts was almost equal to that of keratinocytes when we applied the graft to the animal. An occlusive dressing was quickly placed over the graft to hold it in position and to prevent it from drying out and then the dressing was removed after 7 days. We prepared combinations of gene-transferred keratinocytes and untreated fibroblasts, of untreated keratinocytes and gene-transferred fibroblasts, and of untreated keratinocytes and fibroblasts as control. Skin biopsies were taken from the grafted skin at various time points and subjected to routine immunohistochemical staining using the LH7.2 monoclonal antibody and ultrastructural analysis. To semiquantify *COL7A1* accumulation in basement membrane zone, we converted color images to gray-scale images, and measured fluorescence intensity (arbitrary scale) in basement membrane zone at 10 areas using NIH Image software. The *COL7A1* deposition index was expressed as the mean ± SD from the 10 values.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Baldeschi C, Gache Y, Rattenholl A, Bouille P, Danos O, Ortonne JP *et al.* (2003) Genetic correction of canine dystrophic epidermolysis bullosa mediated by retroviral vectors. *Hum Mol Genet* 12:1897–905

Burgeson RE (1993) Type VII collagen, anchoring fibrils, and epidermolysis bullosa. *J Invest Dermatol* 101:252–5

Chen M, Costa FK, Lindvay CR, Han YP, Woodley DT (2002a) The recombinant expression of full-length type VII collagen and characterization of molecular mechanisms underlying dystrophic epidermolysis bullosa. *J Biol Chem* 277:2118–24

Chen M, Kasahara N, Keene DR, Chan L, Hoeffler WK, Finlay D *et al.* (2002b) Restoration of type VII collagen expression and function in dystrophic epidermolysis bullosa. *Nat Genet* 32:670–5

Christiano AM, Greenspan DS, Hoffman GG, Zhang X, Tamai Y, Lin AN *et al.* (1993) A missense mutation in type VII collagen in two affected siblings with recessive dystrophic epidermolysis bullosa. *Nat Genet* 4:62–6

Christiano AM, Greenspan DS, Lee S, Uitto J (1994a) Cloning of human type VII collagen, complete primary sequence of the alpha 1(VII) chain and identification of intragenic polymorphisms. *J Biol Chem* 269:20256–62

Christiano AM, Hoffman GG, Chung-Honet LC, Lee S, Cheng W, Uitto J *et al.* (1994b) Structural organization of the human type VII collagen gene (COL7A1), composed of more exons than any previously characterized gene. *Genomics* 21:169–79

Ghazizadeh S, Taichman LB (2000) Virus-mediated gene transfer for cutaneous gene therapy. *Hum Gene Ther* 11:2247–51

Heinonen S, Mannikko M, Klement JF, Whitaker-Menezes D, Murphy GF, Uitto J (1999) 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* 112:3641–8

Krueger GG (2000) Fibroblasts and dermal gene therapy. *Hum Gene Ther* 11:2289–96

Ortiz-Urda S, Lin Q, Green CL, Keene DR, Marinkovich MP, Khavari PA (2003) Injection of genetically engineered fibroblasts corrects regenerated human epidermolysis bullosa skin tissue. *J Clin Invest* 111:251–5

Ortiz-Urda S, Thyagarajan B, Keene DR, Lin Q, Fang M, Calos MP *et al.* (2002) Stable nonviral genetic correction of inherited human skin disease. *Nat Med* 8:1166–70

Pulkkinen L, Uitto J (1999) Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol* 18:29–42

Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–43

Roth DA, Tawa NE Jr, O'Brien JM, Treco DA, Selden RF (2001) Factor VIII Transkaryotic Therapy Study Group: nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med* 344:1735–42

Ryynanen J, Sollberg S, Parente MG, Chung LC, Christiano AM, Uitto J (1992) Type VII collagen gene expression by cultured human cells and in fetal skin, abundant mRNA and protein levels in epidermal keratinocytes. *J Clin Invest* 89:163–8

Sawamura D, Yasukawa K, Kodama K, Yokota K, Sato-Matsumura KC, Toshihiro T *et al.* (2002) The majority of keratinocytes incorporate intradermally injected plasmid DNA regardless of size but only a small proportion of cells can express the gene product. *J Invest Dermatol* 118:967–71

Towers GJ, Stockholm D, Labrousse-Najburg V, Carlier F, Danos O, Pages JC (1999) One step screening of retroviral producer clones by real time quantitative PCR. *J Gene Med* 1:352–9

Uitto J, Chung-Honet LC, Christiano AM (1992) Molecular biology and pathology of type VII collagen. *Exp Dermatol* 1:2–11

Uitto J, Pulkkinen L (2001) Molecular genetics of heritable blistering disorders. *Arch Dermatol* 137:1458–61

Vogel JC (2000) Nonviral skin gene therapy. *Hum Gene Ther* 11:2253–9

Woodley DT, Keene DR, Atha T, Huang Y, Ram R, Kasahara N *et al.* (2004) Intradermal injection of lentiviral vectors corrects regenerated human dystrophic epidermolysis bullosa skin tissue *in vivo*. *Mol Ther* 10: 318–326

Woodley DT, Krueger GG, Jorgensen CM, Fairley JA, Atha T, Huang Y *et al.* (2003) Normal and gene-corrected dystrophic epidermolysis bullosa fibroblasts alone can produce type VII collagen at the basement membrane zone. *J Invest Dermatol* 121:1523–747