Targeted Skipping of a Single Exon Harboring a Premature Termination Codon Mutation: Implications and Potential for Gene Correction Therapy for Selective Dystrophic Epidermolysis Bullosa Patients

Maki Goto[1], Daisuke Sawamura[1], Wataru Nishie[1], Kaori Sakai[1], James R. McMillan[1], Masashi Akiyama[1] and Hiroshi Shimizu[1]

This study examined the feasibility of antisense oligoribonucleotide (AON) therapy for dystrophic epidermolysis bullosa (DEB). AON was designed to induce skipping of a targeted exon containing a premature termination codon mutation, resulting in restoration of the open reading frame. We targeted exon 70 of *COL7A1*, as a recurrent mutation 5818delC in Japanese DEB patients was localized to exon 70. We found that one AON induced effective skipping of normal exon 70 containing 16 amino acids. Attachment and migration analyses showed that recombinant collagen without contribution of exon 70 was similar in effect to normal type VII collagen. Next, we synthesized mutation-specific AON by deleting cytosine at 5818. Introduction of this AON into DEB keratinocytes harboring 5818delC showed that the AON induced skipping of exon 70 in the abnormal 5818delC allele. Furthermore, 6.2% of DEB keratinocytes started to express type VII collagen *in vitro* after application of the mutation-specific AON. Injection of the AON into rat model grafted with DEB keratinocytes and fibroblasts induced a low amount of type VII collagen expression. We conclude that skipping of targeted exons using mutation-specific AON may show potential for future gene therapy for DEB patients.

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INTRODUCTION

The transfer of normal genes into somatic cells is one strategy to treat patients with genetic diseases. However, this strategy still encounters problems including efficacy of gene transfer rate and practical clinical safety. Thus, other strategies, including pharmacological therapy or gene correction, are receiving increasing attention.

Recently, studies of muscular dystrophy have demonstrated the feasibility of modulating intron–exon splicing using antisense oligoribonucleotides (AONs), which may induce exon skipping, resulting in slightly shorter, but in-frame, mRNA transcripts (Mann *et al.*, 2001; Lu *et al.*, 2003). In muscular dystrophy caused by mutations in the dystrophin gene, shorter transcripts found in patients with milder phenotypes have a significantly longer life expectancy when compared to the patients with a complete loss of dystrophin expression (Monaco *et al.*, 1988; England *et al.*, 1990). These observations have led to the idea of using AON to skip abnormal, mutated exons to restore the open reading frame and convert a severe phenotype into a milder form (Mann *et al.*, 2001; Lu *et al.*, 2003). The mechanism of exon skipping is based upon AON, small synthetic RNA molecules that are designed to bind to specific sequences within the pre-mRNA (Mayeda *et al.*, 1990; Galderisi *et al.*, 1999).

Dystrophic epidermolysis bullosa (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, and patients generally exhibit tissue separation beneath the lamina densa of the epidermal basement membrane where anchoring fibrils are present in normal skin but structurally compromised in DEB (Fine *et al.*, 2000). DEB is caused by mutations in the *COL7A1* gene encoding type VII collagen, the major component of anchoring fibrils (Uitto *et al.*, 1995; Fine *et al.*, 2000). Several methods have achieved transfer of normal *COL7A1* into the patients' skin (Chen *et al.*, 2002; Ortiz-Urda *et al.*, 2002; Goto *et al.*, 2006), but this has never yet been extended to patients in clinical practice.

Previous report demonstrated an interesting DEB case whose manifestation was milder than expected from mutations in genomic DNA (McGrath *et al.*, 1999). Further mRNA analysis revealed that the mutation led to skipping of that single exon and subsequent maintenance of the open reading

[1]*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan*

Correspondence: Dr Daisuke Sawamura, Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Sapporo 060-8638, Japan. E-mail: smartdai@med.hokudai.ac.jp

Abbreviations: AON, antisense oligoribonucleotide; DEB, dystrophic epidermolysis bullosa; HS-RDEB, Hallopeau–Siemens recessive DEB; RT-PCR, reverse transcriptase-PCR

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frame. In addition, missense mutations are known to provide unexpected *COL7A1* splicing outcomes (Wessagowit *et al.*, 2005). The *COL7A1* mutations causing single exon skipping resulted in milder cases than the predicted Hallopeau–Siemens recessive DEB phenotype (HS-RDEB) with nonsense mutations (Terracina *et al.*, 1998).

In this study, we have examined the feasibility of using AON in targeted exon skipping to modulate *COL7A1* splicing in such a manner that the translational open reading frame can be restored in keratinocytes from DEB patients.

RESULTS

Detection of exon 70 skipping in cultured keratinocytes

We targeted exon 70 for AON therapy as the recurrent premature termination codon mutation 5818delC in DEB patients was localized to exon 70 (Tamai *et al.*, 1999; Sawamura *et al.*, 2005). We synthesized two potential AONs, h70AON1 and h70AON2, which comprised full-length phosphorothioate backbone and HPLC-purified 2′-*O*-methyl-modified ribose molecules (Figure 1a). To evaluate the effects of h70AON1 and h70AON2 on *COL7A1* skipping of normal exon 70, we introduced them to HaCaT keratinocytes or normal human epidermal keratinocytes, and amplified *COL7A1* cDNA by reverse transcriptase-PCR (RT-PCR) with the forward primer on the border of exons 65 and 66, and a reverse primer on exon 72 (66–72 primer set). The h70AON1 samples showed a single 347 bp band. In the h70AON2 experiment, the samples showed a strong upper 347 bp band, whereas the lower 299 bp band was apparent (Figure 1b). Sequence analysis revealed that the upper 347 bp band consisted of exons 66, 67, 68, 69, 70, 71, and 72, whereas a lack of exon 70 was found in the lower 299 bp band. To semiquantify the amount of the lower and upper bands, we subcloned the PCR product to the TA cloning vector. The rate of the lower band to the upper band was expressed as the exon skipping rate. The result showed that the rates in samples of HaCaT cells and normal human keratinocytes were 18.2 and 28.1%, respectively. Each value represents the mean ± SD of four samples.

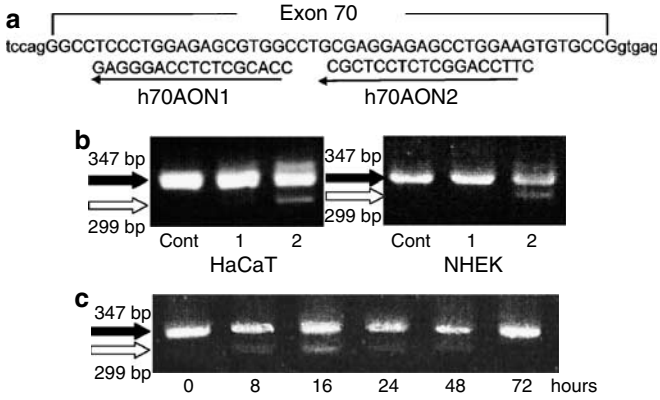

Figure 1. Detection of exon 70 skipping in cultured keratinocytes. The recurrent premature termination codon mutation 5818delC in DEB patients was localized to exon 70. We introduced h70AON1 and h70AON2 into HaCaT keratinocytes or normal human keratinocytes (NHEK), and amplified *COL7A1* cDNA by RT-PCR with the 66–72 primer set. (**a**) Sequences of h70AON1 and 2 are shown. (**b**) The h70AON1 samples (1) showed a single 347 bp band containing exon 70 (black arrow). The h70AON2 samples (2) showed an upper strong 347 bp band with exon 70 (black arrow), whereas the weaker lower 299 bp band without exon 70 (white arrow) was apparent. Only the 347 bp band was found in control samples without AON treatment (Cont). (**c**) We performed a time-course experiment, in which *COL7A1* expression in cultured HaCaT cells was examined 8, 16, 24, 48, and 72 hours after h70AON2 transfer. We found the highest expression of the 299 bp band at the 16 hours after AON transfer although the effect of AON was totally extinct 72 hours after the transfer.

These results indicate that h70AON2 could induce skipping of exon 70. We simultaneously performed a time-course experiment, in which *COL7A1* expression in cultured HaCaT cells was examined 8, 16, 24, 48, and 72 hours after h70AON2 transfer. We found the highest expression of the 299 bp band at 16hours after AON transfer, although the effect of AON was totally extinct 72 hours after the transfer (Figure 1c). We obtained the samples 16 hours after transfer of h70AON2 for further experiments.

Detection of exon 70 skipping *in vivo*

To simulate AON therapy in clinical practice, we transplanted human skin onto a nude rat and then injected 30 μg of h70AON2 to the graft. Saline was injected in a control. After 16 hours, the skin biopsies were taken from the injected site and were subjected to RT-PCR analysis. RT-PCR with the 66–72 primer set amplified a 347 bp strong band containing exon 70 and a weak signal of 299 bp band without exon 70 from the treated sample, whereas the control sample showed 347 bp band alone (Figure 2). We also applied 0.3 or 3 μg of h70AON2 to the graft, resulting in no signal of 299 bp band (data not shown). TA cloning of the 30 μg h70AON2 samples showed that the proportion of the lower band to the upper bands was 12.2%. To verify exon 70 skipping, we synthesized a 70Δ primer set with a forward primer on border of exons 63 and 64, and reversel primer on the border of exons 69 and 71. RT-PCR amplification with the 70Δ primer set detected a 305 bp band in only the AON-treated samples, which were indicative of exon skipping of 70 (Figure 2).

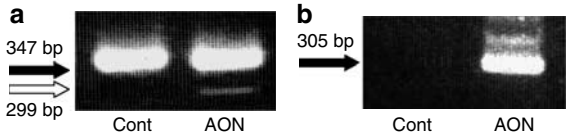

Figure 2. Detection of exon 70 skipping *in vivo*. We transplanted human skin to a nude rat and then injected 30 μg of h70AON2 AON into the graft. After 16 hours, the skin biopsies were taken from the injected site and were subjected to RT-PCR analysis. (**a**) RT-PCR with the 66–72 primer set amplified a 347 bp strong band (black arrow) containing exon 70 and a weaker signal of 299 bp band (white arrow) without exon 70 from the (AON)-treated sample, whereas the control sample showed a 347 bp band alone (Cont). (**b**) RT-PCR amplification with the 70Δ primer set detected a 305 bp band only in AON-treated samples. The AON lane seemed to have the higher molecular weight band in addition to the 305 bp *COL7A1* band. However, subcloning of PCR product to a TA cloning vector (Invitrogen, Carlsbad, CA) could not detect the corresponding *COL7A1* cDNA and we thought it to be a PCR artifact.

Assays for type VII collagen without exon 70

There is a possibility that the deletion of 16 amino acids, which are encoded by exon 70, may abolish some of the functions of type VII collagen. Therefore, we prepared retrovirus vector containing *COL7A1* cDNA without exon 70, and introduced the gene to keratinocytes from HS-RDEB patient who harbored heterozygous *COL7A1* 5818delC and 1474del8 mutations, and showed no expression of type VII collagen in immunofluorescence level as mentioned below (Goto *et al.*, 2006). The Western blot of the supernatant showed that HS-RDEB keratinocytes began to express type VII collagen after gene introduction (Figure 3a). The amounts of secreted collagen were almost identical in culture media between the normal *COL7A1* and *COL7A1*Δ70 samples. Subsequently, the cultured keratinocytes supernatant and the keratinocytes themselves were used for *in vitro* and *in vivo* studies. Cell migration assay using normal keratinocytes showed that the supernatants both with *COL7A1* and *COL7A1*Δ70 samples had a similar effect on the migration of normal keratinocytes (Figure 3b). The cell adhesion assay also demonstrated that *COL7A1*Δ70 supernatant had almost the same adhesive properties for the cells as normal *COL7A1* (Figure 3c). Furthermore, we constructed artificial skin grafts using untreated HS-RDEB fibroblasts and HS-RDEB keratinocytes transduced with *COL7A1* and *COL7A1*Δ70, and then applied them to a nude rat model. Ultrastructural analysis of the grafts showed formation of anchoring fibrils in both samples with *COL7A1* and *COL7A1*Δ70 samples (Figure 4). These results demonstrated that type VII collagen that lacks 16 amino acids of exon 70 might have similar properties to normal type VII collagen, suggesting that exon 70 skipping by AON is promising.

Elucidation of AON specific for 5818delC mutation

We designed mutation-specific AON hm70AON by deleting cytosine at 5818 (Figure 5a). Then we introduced hm70AON to normal human keratinocytes and HS-RDEB keratinocytes and performed the following RT-PCR analysis. The sample from HS-RDEB keratinocytes showed two PCR bands with or without exon 70, whereas a strong upper single band was observed in of the sample from normal human keratinocytes (Figure 5b). This indicated that the hm70AON induced skipping of exon 70 predominantly in abnormal allele with the 5818delC mutation. The sample from HS-RDEB keratinocytes without the AON treatment as control also showed the single band. TA cloning of the PCR samples showed that the exon skipping rates of HS-RDEB and normal keratinocyte samples were 18.5 and 1.3%, respectively; therefore, hm70AON induced skipping of exon 70 predominantly in abnormal allele with 5818delC. This HS-RDEB patient harbored heterozygous *COL7A1* 5818delC and 1474del8 mutations, and RT-PCR analysis using the keratinocyte cDNA showed no naturally occurring skipping of exon 70 containing the frameshift mutation (Figure 5b). Immunofluorescence analysis demonstrated using type VII collagen no expression of type VII collagen (Figure 5c) and ultrastructural study

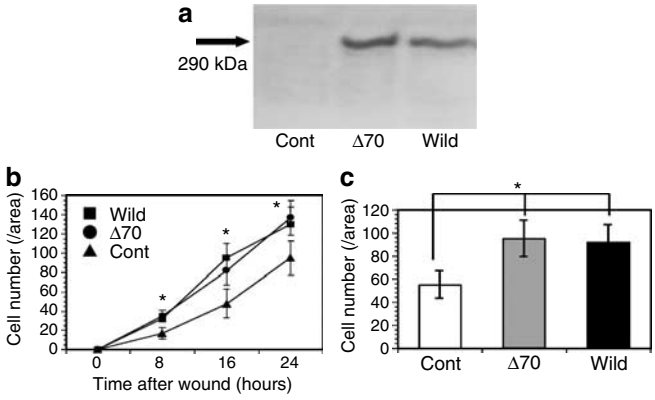

Figure 3. Assays for the type VII collagen without exon 70. We prepared retrovirus vectors with normal *COL7A1* cDNA (Wild) or *COL7A1* cDNA without exon 70 (Δ70), and introduced the gene into DEB keratinocytes, which showed no expression of any type VII collagen. (**a**) The Western blot of the supernatant showed that the amount of secreted type VII collagen in the culture media was almost similar to that of the normal *COL7A1* and *COL7A1*Δ70 samples. Cont: no transfection. (**b**) Cell migration assay showed that the supernatants from both *COL7A1* and *COL7A1*Δ70 samples had a similar effect on normal human keratinocyte migration. (**c**) The cell adhesion assay also demonstrated that *COL7A1*Δ70 supernatant had almost the same adhesive ability for normal keratinocytes as that of *COL7A1* (Figure 3). Each value represents mean ± SD of six samples. *$P<0.01$: significant difference between cont *versus* wild or cont *versus* Δ70 samples.

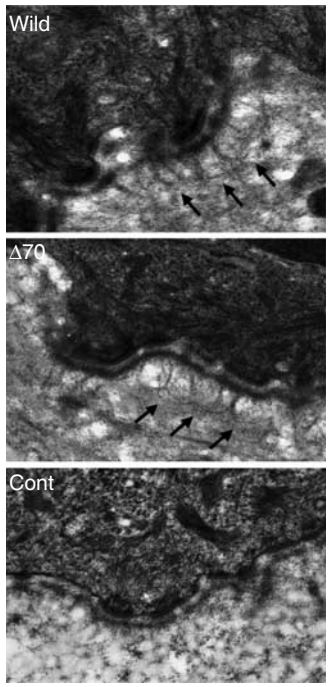

Figure 4. Formation of anchoring fibrils containing COL7A1Δ70. We constructed artificial skin grafts using DEB fibroblasts and keratinocytes transduced with wild-type *COL7A1* or *COL7A1*Δ70, and then grafted them onto a nude rat model. Ultrastructural analysis of the grafts showed that formation of anchoring fibrils could be identified in both samples with *COL7A1*- and *COL7A1*Δ70-transfected cells. Although no obvious anchoring fibrils were observed in control grafts with untransfected DEB keratinocytes alone (Cont), there were wisp-like structures below the lamina densa.

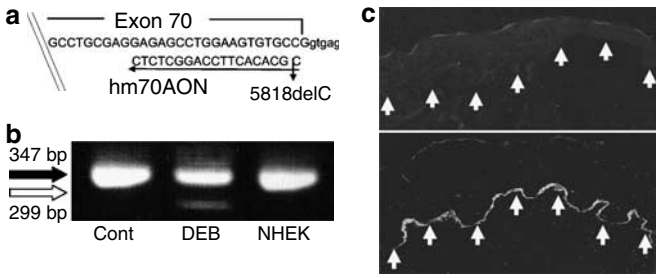

Figure 5. Elucidation of AONs specific for 5818delC mutation. We introduced mutation-specific AON hm70AON into normal human keratinocytes and DEB keratinocytes and performed the following RT-PCR analysis. (**a**) Sequences of mutation-specific AON hm70AON are shown. (**b**) The sample from DEB keratinocytes showed two PCR bands with exon 70 (347 bp) or without exon 70 (299 bp), whereas there was little or no lower band (299 bp) observed in the normal human keratinocytes sample (NHEK). Cont: the sample from DEB keratinocytes without the AON treatment. This HS-RDEB patient harbored heterozygous *COL7A1* 5818delC and 1474del8 mutations and RT-PCR analysis showed no naturally occurring skipping of exon 70 containing the frameshift mutation. (**c**) Immunofluorescence analysis of the patient using type VII collagen antibody demonstrated no expression of type VII collagen (the upper panel). The lower panel shows the expression from normal control. Arrows indicate epidermal–dermal junction.

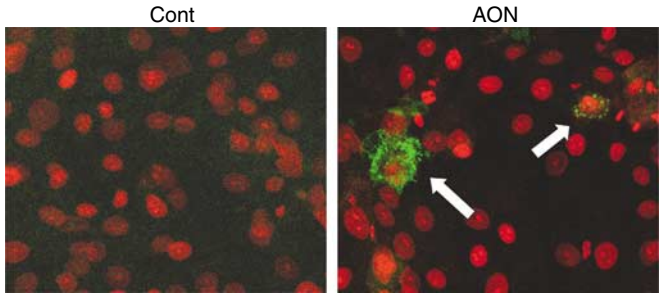

Figure 6. Rescue of type VII collagen expression in DEB keratinocytes *in vitro*. We transfected the hm70AON (AON) into cultured HS-RDEB keratinocytes and stained with an antibody against type VII collagen. Cont: no treatment of AON. Some cells were shown to express type VII collagen after the treatment (arrows).

revealed markedly reduced anchoring fibrils in the basement membrane zone (data not shown).

Rescue of type VII collagen expression in DEB keratinocytes *in vitro*

To examine *COL7A1* expression in the HS-RDEB keratinocytes treated with hm70AON, we transfected hm70AON into cultured HS-RDEB keratinocytes and stained with an antibody against type VII collagen (LH7.2). Some cells were shown to express type VII collagen (Figure 6). The rate of cell expression per total number of cells was 6.2%. We transfected h70AON1 and h70AON2, but no cells showed collagen VII immunoreactivity (data not shown).

Injection of hm70AON to the graft from DEB keratinocytes and fibroblasts

To determine the therapeutic feasibility of AON therapy in clinical practice, we constructed an artificial skin graft using HS-RDEB keratinocytes and fibroblasts, transplanted the graft to a nude rat, and then injected 30 μg of hm70AON to several portions of the graft. Saline was injected as a control. RT-PCR with the 66–72 primer set amplified only 347 bp band containing exon 70 and a weak signal for the 299 bp band without exon 70 from the treated sample (Figure 7a). The 70Δ primer set detected 305 bp band only in AON samples, indicating exon skipping of 70 (Figure 7b). Control samples did not express either 299 bp band by the 66–72 primer set, or 305 bp band using the 70Δ prime set. Furthermore, we performed immunohistochemical analysis of the treated graft with a type VII collagen antibody and could detect definite but intermittent linear dermal–epidermal junction immunoreactivity in the majority of treated samples, whereas no immunoreactivity was observed in control samples (Figure 7c and d).

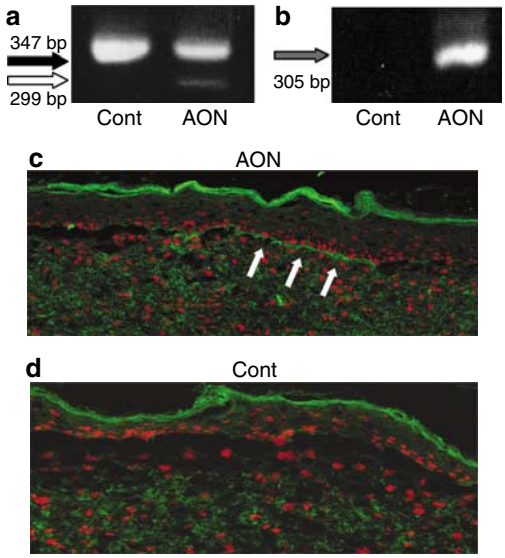

Figure 7. Injection of hm70AON into the graft from DEB keratinocytes and fibroblasts. We constructed an artificial skin graft using HS-RDEB keratinocytes and fibroblasts, transplanted the graft onto a nude rat, and then injected hm70AON into the graft. Saline was injected in a control. (**a**) RT-PCR with the 66–72 primer set amplified a 347 bp band containing exon 70 and a weak signal with the 299 bp band without exon 70 from the treated sample. (**b**) The 70Δ primer set detected only the 305 bp band in AON-treated samples. (**c**) Immunohistochemical analysis of the treated graft with type VII collagen antibody detected discontinuous, linear immunoreactivity along the dermal–epidermal junction (arrows). (**d**) Saline was injected in a control graft. Original magnification (**c**, **d**) ×200.

DISCUSSION

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 study has examined the feasibility of targeted exon skipping using AON. As the intron/exon organization of *COL7A1* contained 118 multiple short exons, deletion of one shorter exon may not significantly interfere with type VII collagen function. In fact, a mutation study revealed that several *COL7A1* splice-site mutations causing skipping of one exon resulted in a milder phenotype than the expected HS-RDEB that harbors nonsense mutations causing a lack of *COL7A1* expression (Terracina *et al.*, 1998). Furthermore, DEB mutations were frequently found in exons encoding collagenous domains and the nucleotide number of these exons is definite multiples of 3. This indicated that abolishment of exons restored an open leading flame of *COL7A1*. These characteristic features of the gene structure encouraged the start of research into the possibility of AON skipping therapy.

COL7A1 mutation database demonstrates presence of recurrent *COL7A1* mutation, and AONs, which are suitable for recurrent mutation, of course, are applicable for more patients than AONs for rare mutation. Thus, this study targeted exon 70 AON therapy, as the recurrent premature termination codon mutation 5818delC in exon 70 was found in more than 20% of recessive DEB sufferers (Tamai *et al.*, 1999; Sawamura *et al.*, 2005). We first synthesized two potential AONs, h70AON1 and h70AON2, for exon 70 and the results indicated that h70AON2 was able to induce skipping of exon 70 *in vitro* and *in vivo*. To understand the effect of exon 70 deletion on the essential functions of type VII collagen, we constructed *COL7A1* cDNA without exon 70 (*COL7A1*Δ70) and introduced this defective gene to HS-RDEB *in vitro* and *in vivo*. The results of attachment and migration analyses showed that the deleted collagen had an apparently similar function to the normal collagen. Next, we examined the ability of the defective collagen to form anchoring fibrils. To eliminate internal expression of type VII collagen, we used keratinocytes and fibroblasts from HS-RDEB who exhibited no *COL7A1* expression and failed to form any anchoring fibril-like structures in patient skin. Ultrastructural examination of the skin graft treated with *COL7A1*Δ70 demonstrated anchoring fibril formations in the sublamina densa. These results indicate that type VII collagen, lacking the 16 amino-acid sequences from exon 70, exhibits a remarkably near-normal assay similar to the normal type VII collagen.

As the sequence of h70AON2 was located at the 5818delC mutation site, we synthesized a mutation-specific AON hm70AON to match the sequence with deletion of 5818 cytosine. After transfection of hm70AON into HS-RDEB harboring the 5818delC defect, RT-PCR demonstrated effective exon 70 skipping. However, as predicted, when this AON was introduced to normal human keratinocytes, we found little skipping against exon 70 in the wild *COL7A1* allele. Some recessive DEB patients harbor heterozygous mutations 5818deC and missense mutations, which would not interfere with the function of this type VII collagen. In this case, hm70AON benefited by not altering the expression of *COL7A1* on the other allele. Further immunofluorescence studies using HS-RDEB cell culture systems showed that transfection of hm70AON could rescue type VII collagen expression in patient's keratinocytes. Approximately, 6% cells began to synthesize type VII collagen.

Finally, we constructed artificial HS-RDEB skin from the patient's keratinocytes and fibroblasts, and introduced hm70AON to the graft. RT-PCR analysis using the 66–72 primer set and the 70*Δ* primer set detected the presence of exon 70 skipping in the treated graft. Furthermore, immunofluorescence using type VII antibody demonstrated the positive expression of type VII collagen along restricted parts of the dermal–epidermal junction. Although we obtained the samples from several specimens and carried out electron microscopic analysis, we could not observe obvious anchoring fibrils ultrastructurally. We might examine no section corresponding to the restricted immunoreactive parts.

We recently suggested that fibroblasts might be a better gene therapy target of DEB treatment than keratinocytes (Goto *et al.*, 2006). The majority of collagen VII *in vivo* are thought to originate from keratinocytes because the level of *COL7A1* expression in the epidermis was much higher than that in the dermis. However, when *COL7A1* expressions of keratinocytes and fibroblast were almost equal after retroviral transfer of *COL7A1*, the gene-transferred fibroblasts supplied a higher amount of collagen VII to the new dermal–epidermal junction than the gene-transferred keratinocytes (Goto *et al.*, 2006). The AON therapy cannot generate an additional *COL7A1* expression, and it just modifies the existing expression. So, we think that AON transferred into keratinocytes gives predominant therapeutic effect in this study.

Finally, disadvantages of this AON approach for DEB are proposed. First, the effect disappeared immediately because AON is easily degraded in the cells. This study showed that the effect of AON was totally extinct 72 hours after the transfer of AON *in vitro*. Second, the method cannot induce the perfect full-length collagen VII. There is the possibility that in-frame exon skipping may lead to a dominant-negative interference. In fact, a 16-bp internal deletion in the *COL7A1* gene leading to in-frame exon skipping caused dominant phonotype of DEB (Cserhalmi-Friedman *et al.*, 1998). Third, transfer efficacy of AON to the skin is relatively low. Kinetic analysis of oligonucleotide topically applied to mouse skin showed early follicular localization, diffusion of the oligonucleotide from the mid-follicle, and subsequent dermal accumulation (Dokka *et al.*, 2005). Our study transfer efficacy was low in our skin graft experiment, but application of AON to actual skin may show a higher transfer efficacy, perhaps owing to the increased presence of skin appendages. In conclusion, we believe that improvement of these points enables this strategy to be applicable to clinical practice.

MATERIALS AND METHODS

AON

We selected exon 70 for AON therapy as the recurrent premature termination codon mutation 5818delC was present in exon 70 in DEB patients (Tamai *et al.*, 1999; Sawamura *et al.*, 2005). We designed a potential AON for exon skipping using the RNA mfold version 3.1 server (Zuker, 2003), and synthesized a full-length phosphorothioate backbone and HPLC-purified 2′-*O*-methyl-modified ribose

molecules. The sequences of the synthesized AONs were as follows: h70AON1, 5′-CCACGCUCUCCAGGGAG-3′ and h70AON2, 5′-CUUCCAGGCUCUCCUCGC-3′ for human type VII collagen: hm70AON, 5′-CGCACACUUCCAGGC-3′ for 5818delC mutation (Figures 1a and 5b).

Cell culture and AON transfection

The human keratinocyte HaCaT cell line was maintained in DMEM with 10% fetal bovine serum. Primary keratinocytes were isolated and grown in the presence of mitomycin C-treated 3T3 feeder layer (Rheinwald and Green, 1975). Briefly, keratinocytes, which were obtained from HS-RDEB patient skin biopsies 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 bovine serum, 5 μg/ml insulin, 10 ng/ml EGFR, 0.4 μg/ml hydrocortisone, and 8 ng/ml cholera toxin. Human fibroblasts were also obtained from a skin biopsy from an HS-RDEB patient and healthy controls, and were cultured in DMEM with 10% fetal bovine serum. This HS-RDEB patient harbored heterozygous *COL7A1* 5818delC and 1474del8 mutations, and showed no expression of type VII collagen (Goto *et al.*, 2006).

After changing of the medium to serum-free Opti-MEM (Gibco Invitrogen, Grand Island, NY), we transfected the AONs into the cells using Lipofectamine 2000 (Gibco Invitrogen) according to the manufacturer's protocols. In all experiments, the cells were exposed to transfection reagent for 16 hours and the media were replaced with fresh growth medium.

Graft experiments

We transplanted artificial and normal skin onto a nude rat. Briefly, 10^6 HS-RDEB fibroblasts were seeded into a collagen sponge scaffold and maintained in DMEM with 10% fetal bovine serum. Confluent cultures containing 10^6 HS-RDEB keratinocytes were treated with dispase (1 nU/ml; Godo Shusei, Tokyo, Japan), and the floating epidermal sheet placed on the collagen sponge. In nude rats (F344/N Jcl-rnu; CLEA Japan, Tokyo, Japan), the sites for transplantation were prepared by excising a 2 cm^2 area of dorsal skin. The collagen sponge containing the fibroblasts was placed into the skin wound and then the epidermal sheet overlaid on the collagen sponge. In other experiments, normal skin samples were obtained from the abdomens of patients undergoing reconstructive plastic surgery at the Hokkaido University Hospital. Each skin sample was placed directly on the 2 cm^2 wound on the nude rat. Afterwards, an occlusive dressing was quickly placed over the graft to hold it in position and to prevent it from drying. After 7 days, the dressing was removed, and then 30 μg AON was diluted in normal saline to a final volume of 150 μl and injected into several portions of the grafts. After 16 hours, the skin biopsies were taken from the injected site and were subjected to RT-PCR analysis as the *in vitro* time–course study indicated the highest expression of the exon-skipping band at 16 hours after AON transfer.

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.

RNA isolation and RT-PCR analysis

Skin samples were taken from the rodent model and were first homogenized using a Polytron homogenizer. Total RNA was extracted from skin samples and cultured cells using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized with reverse transcriptase (Life Sciences, St Petersburg, FL) using an oligo-dT primer. To determine the splice-specific expression of exon 70, we designed the forward PCR primer on the border of exons 65 and 66, and the reversel primer on exon 72. The amplification conditions were carried out using the 66–72 primer set, forward 5′-GAAGGGAGAGAAAGGAGATT-3′ and reversel 5′-GGAAGCTACCAGAGCTCTCA-3′, for 35 cycles of reaction at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. In addition, to verify the skipping of exon 70, we set the forward primer on the border of exons 63 and 64, and reversel primer on the border of exons 69 and 71. The amplification conditions were carried out with the 70Δ primer set, forward 5′-TGGATTACCGGGAAAGCCAG-3′ and reversel 5′-GATCCACATTCTGCTCCCCT-3′, for 35 cycles of reaction at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The PCR products were fractionated on 2% agarose gels and directly sequenced by di-deoxy dye-terminator method using an automated sequencer (ABI Prism Genetic Analyzer 3100, PE Biosystems, Foster city, CA). When several bands were found, we subcloned the PCR sample into a TA cloning vector (Gibco Invitrogen). After transformation of the bacteria, we picked up 50 colonies and examined the inserts to semiquantify the amounts of the bands. The rate of the lower band to the upper band was expressed as the exon skipping rate. Each value represents the mean ± SD of four samples. Moreover, the PCR products were directly sequenced by di-deoxy dye-terminator method using an automated sequencer (ABI Prism Genetic Analyzer 3100, PE Biosystems, Foster city, CA).

Immunostaining and Western blot analysis. Cultured, transfected cells were fixed with 2% paraformaldehyde in phosphate-buffered saline, and were then incubated with the mAb LH7.2 against the NC1 domain of collagen type VII (Chemicon, Temecula, CA). The secondary antibody was FITC-conjugated goat anti-mouse IgG and preparations were examined under a fluorescence microscope. Subconfluent keratinocyte 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 protein concentration and desalting. The samples were separated on a 5% polyacrylamide gel under reducing conditions. Immunoblotting analysis was performed using the LH7.2 mAb followed by the secondary antibody goat anti-mouse IgG conjugated to peroxidase. The resultant complexes were processed for Phototope horse radish peroxidase Western Blot Detection System (Cell Signaling, Beverly, MA) according to the manufacturer's protocol.

Assays for the type VII collagen without exon 70

Introduction of h70AON2 into keratinocytes induced skipping of exon 70 (see the Results section). We prepared cDNA from the introduced keratinocytes and amplified cDNA without exon 70 (*COL7A1*Δ70). A retroviral vector pDON(*Δ*) was created by removing the Simian virus-40 promoter and *Neo* gene from pDON-AI (Takara, Kyoto, Japan). The genes *COL7A1* and *COL7A1*Δ70 were inserted into pDON(Δ) and these retrovirus plasmids were introduced into the amphotropic amphopack-293 packaging cells (Clontech, Palo Alto, CA) using calcium phosphate co-precipitation.

The viral particles were recovered from the cell culture medium 48 hours later and applied to cultured DEB keratinocytes that failed to express *COL7A1*. The supernatant of the cultured keratinocytes and the keratinocytes themselves were used for *in vitro* and *in vivo* studies.

The methods for the cell migration assay and cell adhesion assay were performed as described previously (Tsuda *et al.*, 2002). Briefly, culture dishes were treated with conditioned supernatants from cultured HS-RDEB keratinocytes transduced with *COL7A1* and *COL7A1*Δ70 for 24 hours. Control dishes were treated with culture medium. After the plating of normal keratinocytes for 48 hours on the treated dishes, the cells were scraped off using a 200 μl yellow pipette tip. Subsequently, at 8, 16, and 24 hours, the number of cells that had moved from the base line into the scratched area was measured. In addition, normal keratinocytes were allowed to attach for 1.5 hours on the treated dishes and, after removal of unattached cells, washed and fixed with 70% ethanol for 10 minutes. Adherent cells were then stained with crystal violet and the number of the cells was measured.

The collagen sponge containing HS-RDEB fibroblasts was placed on skin wound of nude rat and the confluent cultures of 10^6 HS-RDEB keratinocytes transduced with *COL7A1* and *COL7A1*Δ70 were overlaid on the collagen sponge, as mentioned above. After 8 weeks, the skin biopsies were taken from the graft and subject to routine ultrastructural analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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