Traceless Targeting and Isolation of Gene-Edited Immortalized Keratinocytes from Epidermolysis Bullosa Simplex Patients

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Epidermolysis bullosa simplex (EBS) is a blistering skin disease caused by dominant-negative mutations in either KRT5 or KRT14, resulting in impairment of keratin filament structure and epidermal fragility. Currently, nearly 200 mutations distributed across the entire length of these genes are known to cause EBS. Genome editing using programmable nucleases enables the development of ex vivo gene therapies for dominant-negative genetic diseases. A clinically feasible strategy involves the disruption of the mutant allele while leaving the wild-type allele unaffected. Our aim was to develop a traceless approach to efficiently disrupt KRT5 alleles using TALENs displaying unbiased monoallelic disruption events and devise a strategy that allows for subsequent screening and isolation of correctly modified keratinocyte clones without the need for selection markers. Here we report on TALENs that efficiently disrupt the KRT5 locus in immortalized patient-derived EBS keratinocytes. Inactivation of the mutant allele using a TALEN working at sub-optimal levels resulted in restoration of intermediate filament architecture. This approach can be used for the functional inactivation of any mutant keratin allele regardless of the position of the mutation within the gene and is furthermore applicable to the treatment of other inherited skin disorders.

INTRODUCTION
Keratinopathies, such as epidermolysis bullosa simplex (EBS), epidermolytic ichthyosis (EI), and pachyonychia congenita (PC), are a group of inherited skin disorders mostly resulting from dominant-negative mutations in genes encoding keratins. Keratins are cytoskeletal proteins expressed in pairs that form heteropolymeric intermediate filaments (IFs), which protect cells from mechanical stress.¹ EBS, which is characterized by intraepidermal blistering after mechanical stress, is due to approximately 110 and 80 mutations in the keratin 5 (KRT5) and keratin 14 (KRT14) genes, respectively.² Keratin 5 (K5), a type II IF protein, and K14, a type I IF protein, are expressed in the basal layer of the epidermis,³ which is home to epidermal stem cells responsible for tissue regeneration and repair.⁴ Depending on the location within the genes, mutations in KRT5 or KRT14 cause different severity subtypes of EBS.⁵,⁶ The mutant keratins polymerize with wild-type (WT) keratins into abnormal filaments that collapse upon mild mechanical stress, forming aggregates resulting in cytolysis and skin blistering.⁷,⁸ The impairment of IF structure and aggregate formation upon stress can also be seen in cultured keratinocytes from EBS patients.⁹⁻¹¹ There is no cure for EBS and other keratinopathies, and current treatments focus on wound management and alleviating symptoms to improve the patient’s quality of life.

One strategy for the treatment of dominant keratinopathies is the elimination of the mutant keratin in epidermal keratinocytes. It is known that keratins are expressed from both alleles and that one functioning allele is sufficient to maintain a structurally stable epidermis.¹²⁻¹⁴ The use of small interfering RNA (siRNA) has shown promise in pre-clinical and clinical studies for EBS and PC.¹⁵⁻¹⁸ However, siRNA cannot fully suppress gene expression and may have poor specificity.¹⁹,²⁰ Additionally, such an approach would not provide a permanent cure, and for successful in vivo application, an efficient and non-invasive delivery method is needed.²¹

An attractive alternative that would provide a permanent cure relies on the use of programmable nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided nucleases (RGNs) derived from the bacterial CRISPR-Cas system. These platforms have revolutionized genetic engineering and allow for the introduction of precise changes to specific sequences within a genome, showing great potential for the treatment of monogenic diseases.²²,²³ By introducing DNA double-strand

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breaks (DSBs), programmable nucleases are able to activate endogenous repair machinery, resulting in gene disruption via non-homologous end joining (NHEJ) or gene correction via homology-directed repair (HDR) in the presence of a donor template. We hypothesize that gene editing can be used to eliminate the mutant protein by disrupting the mutant allele while leaving the WT allele intact. EBS can thus serve as a model for the development of gene therapy approaches for this type of dominant-negative diseases. Furthermore, for several reasons, such an approach has a high potential to be translated into the clinic. First, the skin is accessible. Second, the epidermis hosts epidermal stem cells that can be grown in culture. Third, these stem cells have the rare ability among adult stem cells to grow clonally ex vivo while maintaining their stem cell capabilities. This property allows the expansion of large numbers of keratinocytes from small skin biopsies. Expanded keratinocytes cannot only serve for the autologous treatment of third-degree burns, a technique that has been widely used for more than 30 years, but have, in addition, been successfully used for combined gene and cell therapies.

Previously, we have shown that ZFNs can be used to efficiently inactivate a transgene in murine epidermal stem cells without affecting stem cell characteristics such as self-renewal and differentiation. The therapeutic effect of the epidermal stem cell strategy depends on the quality of the cells used for transplantation because only self-renewing stem cells sustain long-term skin regeneration. Culturally stressed epidermal stem cells may differentiate, possibly resulting in early graft loss. Culture stress may result from extended growth in culture and the use of selective methods such as fluorescence-activated cell sorting (FACS) and, possibly, antibiotic selection. We therefore aimed to develop an approach for the screening and isolation of edited keratinocytes without the use of selection markers, minimizing culture stress and permitting the isolation of keratinocytes with desired changes.

A straightforward strategy would be the use of engineered nucleases targeting the disease-causing point mutations for allele-specific inactivation. However, this is difficult to implement for clinical translation because, for every single dominant-negative mutation, novel programmable nucleases with varying efficiencies and specificities would have to be designed and thoroughly validated. Although the simplicity of engineering behind the CRISPR/Cas9 system, along with highly specific Cas9 variants, may overcome this issue, the number of mutations that can be targeted is restricted by the protospacer adjacent motif (PAM) sequence. Furthermore, several of the known point mutations are not suitable for this strategy because targeting certain regions of the coding sequence may result in evasion of nonsense-mediated mRNA decay (NMD) and expression of a truncated protein rather than elimination of protein expression. An alternative strategy that can be used for all dominant-negative mutations and decreases the probability of producing truncated proteins is based on non-allele-specific targeting of the 5' end of a coding sequence using a nuclease that displays unbiased monoafllic gene disruption events. Because of unbiased targeting of a non-allele-specific nuclease, a subset of cells treated with this nuclease will contain frameshifting indel mutations (small insertions or deletions) only on the mutant allele. These cells can then be identified using a screening approach and clonally expanded for clinical use. We sought to use TALENs because they combine high cleavage activity with high specificity. TALENs function as dimers, and their activity depends on the spacer length between the monomer binding sites. We therefore assessed four TALEN pairs that target binding sites with spacers of 12–15 bp to identify TALENs displaying high monoafllic gene disruption.

The present study describes a novel approach for the screening and clonal expansion of keratinocytes edited with a TALEN of lower activity that displayed unbiased monoafllic gene disruption. Disruption of the mutant KRT5 allele in immortalized patient-derived keratinocyte clones eliminated structural abnormalities in IFs and formation of IF aggregates upon stress. Therefore, a less active engineered nuclease combined with screening and clonal expansion can be used for the development of ex vivo gene therapies of autosomal dominant keratinopathies, such as EBS, regardless of the position of the mutation within the gene.

RESULTS
Targeting of the KRT5 Locus
We generated six TALEN monomers to target two distinct sites in exon 1 of KRT5 (Figure 1A) and tested them in pairs for their activity in immortalized normal and EBS human keratinocytes with missense mutations in exon 2 and exon 7 (Figure 1B) using an optimized screening approach (Figure 1C). The screening approach focused on determining the gene disruption efficiencies, followed by clonal expansion and subsequent screening of transfected clones. The edited clones were then transferred to a larger culture vessel for additional expansion, followed by further analysis. TALENs 1A-2 and 1B-2 were composed of different left monomers, “1A” or “1B,” and a common right monomer, “2.” These two TALEN pairs targeted the same region with a difference in monomer spacing of 12 bp and 14 bp, respectively. Similarly, TALENs 3-4A and 3-4B had a common left monomer, “3,” and targeted the same region with a monomer spacing of 13 bp and 15 bp, respectively. The presence of CpG dinucleotides within the TALEN target sites was noted because potentially methylated cytosines might affect TALEN binding (Figure S1).

Gene disruption efficiencies of TALENs were assessed by the detection of indels resulting from imperfect repair via NHEJ using the T7E1 assay. Cultured immortalized normal (NKn21) and EBS (EB11 and EB21) keratinocytes were cotransfected with two plasmids, each encoding a TALEN monomer. Activity of all four TALEN pairs was detected in NKn21, EB11, and EB21 cells (Figure 2). In immortalized normal human keratinocytes (NKn21) transfected with TALEN 1A-2, 36% of alleles contained indels. The same TALEN induced indels of 25% and of 18% in EB11 and EB21, respectively. A consistent lower activity was observed in cells transfected with TALEN pairs 1B-2 (29% in NKn21, 22% in EB11, and 16% in EB21), 3-4A (22% in NKn21, 21% in EB11, and 16% in EB21), and 3-4B (18% in NKn21, and 6% in EB11 and EB21) compared with cells transfected with TALEN 1A-2, with TALEN 3-4B displaying the lowest levels of NHEJ.
We next tested whether the TALENs could achieve similar gene disruption efficiencies in primary human keratinocytes isolated from a healthy donor. Primary human keratinocytes were edited by all four TALEN pairs with efficiencies similar to EB21 (Figure 2). The different levels of NHEJ detected between the cell lines were due to varying transfection efficiencies as shown by flow cytometric analysis (Figure S2). Lower transfection efficiency was observed for the EBS lines (55% for EB11 and 47% for EB21) and primary keratinocytes (43%) compared with the normal keratinocyte line (68% for NKc21). Analysis of transfection efficiencies using flow cytometry data of primary keratinocytes showed the presence of three populations that differed in size, with two smaller populations containing 47% and 48% transfected (EGFP+) cells, whereas only 25% of the larger cells were transfected (Figure S3). This result suggests that the overall decreased transfection efficiency of primary keratinocytes was due to the presence of larger (i.e., terminally differentiated) postmitotic keratinocytes abundant in primary cultures, which are known to be more difficult to transfect. In summary, analysis of four different TALEN pairs in patient-derived keratinocytes showed varying activities.

**Screening for Monoallelic Edited Keratinocyte Clones**

To determine the number of edited clones, immortalized EBS keratinocytes transfected with the different TALEN pairs were clonally expanded, and TALEN target sites were analyzed for indel formation using direct PCR and sequencing. Screening of keratinocyte clones revealed the presence of distinct indels at the TALEN target sites (Figure 3A; Figure S4). The most common indels observed were deletions, and the majority of the NHEJ events resulted in frameshifts.
Interestingly, two independent indel profiles were observed in the same target site of some clones edited with TALEN 1A-2 or 3-4A (Figure S4), suggesting the occurrence of multiple NHEJ events in the same cell that were generated from repeated TALEN binding and cleavage. Indels were detected in 29% of EB11 clones treated with TALEN 1A-2 (Figure 3B). Similarly, indels were detected in 25% of EB21 clones treated with TALEN 1A-2 (Figure 3C). Because of the similarity in the levels of gene disruption in immortalized keratinocytes treated with TALEN 1B-2 or 3-4A, cells treated with TALEN 1B-2 were not seeded for clonal expansion and further analysis. TALEN 3-4A resulted in editing frequencies of 23% in EB11 and 21% in EB21 clones, whereas TALEN 3-4B induced indel formation of 9% and 7% in EB11 and EB21 clones, respectively.

TALEN 1A-2 produced a surprisingly high number of biallelic modifications in both EB11 and EB21 clones (Figures 3D and 3E). Both biallelic and monoallelic modifications were seen in EB11 and EB21 clones transfected with TALEN 3-4A. Interestingly, the ratio of biallelic to monoallelic modifications between EB11 and EB21 clones varied. Edited EB11 clones exhibited 75% of biallelic and 25% monoallelic disruption, whereas EB21 clones exhibited 54% biallelic and 46% monoallelic disruption. All EB11 and EB21 clones edited with TALEN 3-4B showed only monoallelic indel formation. Clones edited on a single allele were further analyzed by allele-specific PCR.

Figure 2. Efficient TALEN-Mediated Disruption of the KRT5 Locus in Human Keratinocytes

NKc21, EB11, EB21, and primary keratinocytes were transfected with expression plasmids encoding the indicated TALEN (1A-2, 12-bp spacer; 1B-2, 14-bp spacer; 3-4A, 13-bp spacer; 3-4B, 15-bp spacer). Genomic DNA was extracted 1 week after transfection and subjected to PCR amplification of the target site, followed by T7E1 assay. The spacer lengths between each TALEN monomer and the extent of cleavage are indicated below. Arrowheads indicate the position of the cleaved and uncleaved products with the corresponding sizes.

Figure 3. Efficient TALEN-Mediated Editing of KRT5 in Immortalized Human Keratinocytes

(A) Sequencing data of edited keratinocyte clones summarizing the types of indels observed; deletions (black) and insertions (white) are shown. Shown are the levels of total insertions (white) and deletions (black) observed; 6% insertions, 94% deletions. Also shown are the levels of frameshift (FS) and non-frameshift (non-FS) NHEJ events; 77% FS, 23% non-FS. (B and C) The percentages of edited (B) EB11 clones transfected with TALEN 1A-2 (29%, n = 34), 3-4A (23%, n = 71), or 3-4B (9%, n = 22) and (C) EB21 clones transfected with TALEN 1A-2 (25%, n = 16), 3-4A (21%, n = 124), or 3-4B (7%, n = 30). (D and E) Allelic disruption frequencies in (D) EB11 clones edited by TALEN 1A-2 (100% bi, n = 10), 3-4A (75% bi and 25% mono, n = 16), or 3-4B (100% mono, n = 2) and (E) EB21 clones edited by TALEN 1A-2 (75% bi and 25% mono, n = 4), 3-4A (54% bi and 46% mono, n = 26), or 3-4B (100%, n = 2). Edited clones with biallelic (gray) and monoallelic (white) modifications are indicated.
and sequencing. All of the edited EB11 clones showed an equal ratio of allelic disruption frequency (Figure 4). In EB21 clones, however, a preference for the WT allele with a disruption ratio of 4:1 was observed; i.e., modification of four WT alleles for every mutant allele. In summary, screening of keratinocytes clones identified clones with corrective modifications and TALEN pairs displaying monoallelic gene disruption.

Editing of Individual Alleles Results in Alterations in Intermediate Filament Structure

To confirm that editing of the KRT5 locus resulted in changes in IF stability, we analyzed two randomly selected EB11 and EB21 clones with indels on either the mutant or the WT allele resulting in frame-shifts (Figure 4). Two EB11 clones, one with a 2-bp deletion on the mutant allele (EB11 Dmut clone, E26) and one with a 4-bp deletion on the WT allele (EB11 DWT clone, 8), resulting in the induction of premature translation-termination codons (PTCs) in two different frames, were analyzed by immunofluorescence. Similarly, two EB21 clones, one with a 2-bp deletion on the mutant allele (EB21 Dmut clone, B77) and one with a 2-bp deletion on the WT allele (EB21 DWT clone, 43), were analyzed by immunofluorescence. Unmodified EB11 and EB21 lines and isolated unmodified clones were used as controls.

Prior to immunofluorescence, keratin filament stability was challenged by the application of thermal or hypo-osmotic stress. Following stress, both immortalized normal and EBS keratinocytes rounded up, suggesting filament disassembly. Additional changes occurred in the EBS cells, indicating reduced resilience of the IF cytoskeleton. Immunofluorescence staining for K5 in EB11 cells showed the presence of cytoplasmic keratin aggregates localized mostly to the cell periphery in 30.3% ± 4.1% of unstressed, 37.3% ± 5.2% of thermally stressed, and 44.5% ± 3.7% of osmotically stressed cells, confirming the severity of the phenotype (Figure 5). Staining of an unmodified EB11 clone showed collapse of filaments forming a shell around the nucleus rather than keratin aggregates in the cell periphery. Analysis of the EB11 Dmut clone E26 showed homogeneous distribution of IF throughout the cytoplasm and absence of keratin aggregates or collapsed filaments around the nucleus. The cells appeared morphologically normal, suggesting that inactivation of the mutant allele restored IF stability. In contrast, EB11 DWT clone 8 showed a more severe phenotype with absence of normal filaments and the presence of a high number of keratin aggregates of various sizes distributed throughout the cytoplasm in 98.6% ± 1.4% of unstressed, 98.1% ± 1.9% of thermally stressed, and all osmotically stressed cells. Additionally, large clumps of collapsed keratin filaments adjacent to the nucleus were observed. Immunofluorescence staining for K5 in EB21 cells did not show the presence of keratin aggregates, which is consistent with the moderate disease phenotype associated with the underlying mutation (Figure S5). Staining of the unmodified EB11 clone showed the absence of a homogeneous cytoskeleton. Similarly to EB11 Dmut clone E26, analysis of EB21 Dmut clone B77 showed an overall improved appearance of the cytoskeleton in all cells. Staining of EB21 DWT clone 43 showed the presence of keratin aggregates in only 5.6% ± 1.1% of thermally stressed and 4.8% ± 0.2% osmotically stressed cells. Immunofluorescence analysis of the edited EBS clones showed that

![Table](image-url)

Figure 4. EB11 and EB21 Monoallelic Clones
Sequencing summary of EB11 (n = 6) and EB21 (n = 15) clones with monoallelic modifications generated by TALEN 1A-2, 3-4A, or 3-4B. Target sites (blue) of TALENs 1A-2, 3-4A, or 3-4B are shown, with thymidine position 0 (red) indicated. N/D, not determined.
inactivation of the mutant KRT5 allele resulted in changes to IF distribution, suggesting restored filament stability. In contrast, inactivation of the WT allele resulted in exacerbation of IF abnormalities. Clones were thermally stressed and visualized using fluorescent microscopy to observe changes in IF stability in real time (Figure 6A). Live-cell imaging was performed by transfecting clones with expression constructs coding for murine keratin 10 (K10) fused to EGFP (Figure 6B). The type I keratin K10 is normally not expressed in keratinocytes grown in culture (i.e., undifferentiated keratinocytes), but will, because of the promiscuity of keratins, integrate into any keratin filament, such as K5/K14 filaments, upon transfection into these cells. No aggregate formation was observed in NKc21 cells after the application of thermal stress (Figure 6C). However, real-time keratin aggregate formation and filament collapse were observed in EB11 cells after the application of thermal stress. In EB11 Δmut clone E26, no aggregate formation was detected in response to thermal stress, suggesting increased stability of keratin filaments. In contrast, EB11 ΔWT clone 8 revealed filament collapse into one single clump around the nucleus. No aggregate formation was observed in EB21 cells (Figure S6). EB21 Δmut B77 clone showed improvements to filament stability, whereas EB21 ΔWT clone 43 showed the presence of keratin aggregates in thermally stressed cells. Observed changes in IF stability and aggregate formation suggest that TALEN-mediated editing of the mutant or WT allele resulted in alleviation or aggravation of the EBS phenotype in vitro, respectively.

To determine whether editing of the KRT5 locus resulted in reduction in K5, protein levels were analyzed using western blotting. Expression levels of K5 were unaltered in the EB11 and EB21 clones (Figures S7A and S7B) compared with the unedited lines, and truncated proteins were not detected. Interestingly, the EB21 line showed higher levels of K5 than NKc21 and EB11, suggesting polyploidy of chromosome 12, which is consistent with the 4:1 ratio of editing, sequencing, and imaging results.

An overview of the modification approach based on EB11 transfected with TALEN 3-4A showed that, of the total population, 5.6% (4 of 71) had monoallelic modifications, and about half of these clones were edited on the mutant allele, as expected from unbiased targeting (Table 1). Taking into account that only two-thirds of all NHEJ events would lead to a frameshift, ~2% of clones would contain a "therapeutic" modification. This is due to the high incidence of biallelic gene disruption. EB11 transfected with TALEN 3-4B, on the other hand, showed 9% (2 of 22) of clones with monoallelic disruption and, thus, 3% of clones potentially carrying a corrective modification.

Off-Target Analysis
An important factor in the successful application of designer nucleases in a clinical setting is specificity. We therefore assessed the off-target activity of each TALEN pair in the transfected EB11 and EB21 populations using a T7E1 assay. Assessment of a total of ten hetero- and homodimeric off-target sites (two to four per TALEN) showed undetectable off-target cleavage in both EB11 and EB21 (Figure S8). Although the T7E1 digestion patterns of some off-target
regions (OT-5, OT-8, OT-9, and OT-10) showed several DNA fragment sizes, including bands in the range of the predicted fragments, these were most likely not specific because exactly the same patterns were also present in the negative controls.

**DISCUSSION**

TALENs hold great potential for the development of ex vivo gene therapies for dominant-negative genetic diseases such as EBS. They enable targeting of stem cells and disruption of the mutant allele, thus providing a permanent cure. We describe an approach for screening and isolation of gene-edited immortalized human epidermal keratinocytes. With this approach, correctly modified clones can be identified in less than 1 month after transfection. The application of such an approach in combination with TALENs displaying unbiased monoallelic gene disruptions in a clinical setting is clinically relevant for several reasons. First, the approach relies on NHEJ, which is significantly more efficient than HDR, especially in primary human keratinocytes (~9% versus <1%).

Second, marker-dependent selection methods such as FACS or antibiotic treatment, which may leave marks in targeted cells and be a source of culture stress for epidermal stem cells, are avoided. Third, epidermal stem cells can be cultured for at least 180 population doublings on feeder cells or up to passage 20 using transforming growth factor β (TGF-β)/BMP inhibitors. Human epidermal stem cells cultured on mouse feeder fibroblasts have been used for both cell and gene therapy,

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**Figure 6. Live Visualization of Keratin Filament Stability in Edited EB11 Clones**

(A) Thermal stress was applied for 30 min at 43°C. Cells were visualized for 4 hr before, during, and after stress. Photographs were taken every 5 min at four fixed positions for each cell line or clone. (B) K5, a type II intermediate filament, and K14, a type I, dimerize to form heteropolymorphic intermediate filaments. K10, also a type I, can dimerize with K5 and be integrated into pre-existing filaments. Labeling of intermediate filaments can be achieved by introducing an EGFP-K10 fusion protein into the cells. (C) Live-cell imaging of NKc21, EB11, EB11 Δmut clone E26, and EB11 ΔWT clone 8 using EGFP-K10 labeling of keratin filaments. K10 will integrate into pre-existing keratin filaments made up of K5 and K14. Keratinocytes were transfected with constructs coding for an EGFP-K10 fusion protein and visualized using fluorescence microscopy. Keratin aggregate formation (white arrows) is indicated. Scale bar, 100 μm.
In our study, we describe traceless targeting of immortalized human epidermal keratinocytes using TALENs. We show that TALENs efficiently disrupted the KRT5 locus in immortalized human epidermal keratinocytes, with 29% of clones showing indels. This is important because increasing the number of modified stem cell clones increases the number of potential candidates for transplantation. We observed similar or higher gene disruption efficiencies in immortalized patient-derived and primary keratinocytes than the previously reported efficiencies in primary fibroblasts from a patient with recessive dystrophic epidermolysis bullosa. We previously described variations in gene disruption efficiencies of TALENs depending on linker and spacer lengths. Here we observed that a TALEN with a 17-amino acid (aa) linker exhibited high efficiency of indel production on a target site with a short optimal spacer of 12 bp. This high cleavage activity was associated with a high frequency of biallelic gene disruption, which would be ideal for gene therapies where complete ablation of a gene is beneficial, such as the expression of CCR5 in HIV-positive individuals. In contrast, a TALEN recognizing a target site with a longer spacer of 13 bp or 15 bp exhibited lower activity, which was associated with more monoallelic gene disruption. Although the overall activity of the 3-4B TALEN pair (15 bp) was lower compared with TALEN 3-4A (13 bp), the absolute frequency of monoallelically modified clones was higher. In the case of EB5, where a functioning WT allele is essential, one could increase the probability of identifying clones with monoallelic modifications by designing TALENs with lower overall activity, but a systematic approach is needed to determine whether these observations remain true for all TALENs. Furthermore, we show that TALENs were able to target the KRT5 locus with high specificity because no off-target cleavage was detected. This is consistent with the previously observed TALEN specificities.

An alternative is to exploit a point mutation or a SNP to specifically target an allele. Efficient targeting of human keratinocytes for therapy is feasible; however, because of the importance of having as many viable epidermal stem cell clones as possible, it would be more strategic to use SNPs or point mutations to target individual alleles because the high specificity of TALENs permits that. However, this would be challenging and time-consuming to implement for 200 mutations in two genes. We only examined spacers of 12–15 bp as opposed to 20–22 bp because this was the highest peak of TALEN activity of the current architecture. Interestingly, sequencing data from clones edited by TALEN 1A-2 or 3-4A suggest multiple consecutive NHEJ events, possibly because of repeated TALEN binding and cleavage. As previously shown, TALENs of the Δ135/+17 architecture are active at a 10-bp spacer between monomers. A Δ2-bp deletion in the spacer of a TALEN originally designed with a 12-bp spacer (e.g., 1A-2) may thus permit a further increase in activity. Similarly, a TALEN originally designed with a 14-bp spacer (e.g., 3-4A) may also be active at the same target site when a Δ2 bp deletion is present.

Furthermore, we show that inactivation of the mutant KRT5 allele resulted in elimination of structural abnormalities in IFs and formation of aggregates upon stress. No previous work has been done for EB5 and other keratin disorders using programmable nucleases. Because of the low availability of primary cells from patient samples, we chose immortalized EB5 keratinocytes as the model system to validate our approach. Furthermore, the two cell lines used were previously characterized and reported to show keratin aggregates in culture. Keratin aggregates are a direct indication of IF abnormalities and phenotype severity because there is a strict genotype-phenotype correlation. Interestingly, EB21 cells were reported to show keratin aggregates in over 10% of stressed cells. However, we only observed keratin aggregates in 5% of stressed cells in the EB21 ΔWT clone. Furthermore, we observed different frequencies of biallelic and monoallelic targeting between EB11 and EB21, with TALEN targeting in EB21 clones showing a preference for the WT allele. Taken together with the observed higher levels of K5 expression, this suggests polyploidy for chromosome 12 in EB21. This is consistent with a previous study that reported tetraploidy in metaphase spreads of EB21 cells. This highlights the importance of choosing the right model system. We observed no reduction in the expression levels of K5 in modified clones. This may be due to upregulation of gene expression from the WT allele.

Overall, our data support that TALENs are excellent tools for gene disruption and have high potential for clinical use. We describe a strategy that is based on a less active TALEN pair that can be used for unbiased monoallelic gene disruption in immortalized patient-derived keratinocytes, followed by screening and isolation of gene-edited immortalized keratinocytes without the use of selection markers. Importantly, inactivation of the mutant KRT5 allele resulted in elimination of IF abnormalities. Together, these findings bring us a step closer to realizing a traceless ex vivo gene therapy for disorders resulting from dominant-negative mutations, such as EB5.

### Table 1. Summary of the Clonal Analysis

| Clones | TALEN Analyzed | Modified | Biallelic | Monoallelic | WT | Mutant |
|--------|----------------|----------|-----------|-------------|-----|--------|
| EB11   | 1A-2           | 34       | 10        | 10          | –   | –      |
| EB11   | 3-4A           | 71       | 16        | 12          | 4   | 1 2    |
| EB11   | 3-4B           | 22       | 2         | –           | 2   | 1 1    |
| EB21   | 1A-2           | 16       | 4         | 3           | 1   | –      |
| EB21   | 3-4A           | 124      | 26        | 14          | 12  | 9 2    |
| EB21   | 3-4B           | 30       | 2         | –           | 2   | 2 –    |

that rely on mouse feeder layers have been approved by the Food and Drug Administration (FDA) (Epigel by Sanofi Biosurgery). Fourth, the fact that programmable nucleases are capable of targeting sites with several mismatches clearly shows that a clonal approach provides a higher degree of safety. It allows the isolation, selection, and transplantation of safe and correctly modified keratinocyte clones from a mass culture.
MATERIALS AND METHODS

Cell Culture and Transfection

Immortalized patient-derived EBS (EB11 and EB21) and healthy donor (NKc21) keratinocyte lines were cultured in Epilife medium (Life Technologies) supplemented with human keratinocyte growth supplement (HKGS, Life Technologies) and penicillin/streptomycin/amphotericin (PSA) (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B; Lonza). EB11 was originally derived from a patient with generalized severe EBS (formerly known as Dowling-Meara EBS, DM-EBS) resulting from a p.Glu475Gly substitution in exon 7.11 EB21 was originally derived from a patient with moderate EBS (formerly known as Koebner-type EBS) resulting from a p.Val186Leu substitution in exon 2.37 NKc21 is a normal keratinocyte line isolated from a healthy donor that was used as a control.20 Primary human keratinocytes were isolated from adult foreskin samples normally discarded after surgical procedures and cultured in CnT-Prime medium (CELLnTEC) supplemented with PSA (Lonza). 3T3-J2 mouse fibroblast feeders were grown in DMEM with 4.5 g/L glucose and L-glutamine (Lonza) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and penicillin/streptomycin (PS) (100 U/mL penicillin and 100 µg/mL streptomycin, Lonza). Cells were maintained at 37°C in a 5% CO2 atmosphere. Cells were cultured in 6-well plates and transfected at 50%–70% confluence with 3 µg of TALEN expression plasmids using 0.5 µL Xfect (Clontech) according to the manufacturer’s instructions. Cells were cultured at 30°C for 3 days 4 hr after transfection as described previously.21 For the assessment of transfection efficiency, cells were transfected with 3 µg of EGFP expression plasmids (Clontech). Cells were cultured at 37°C and analyzed for EGFP expression 48 hr after transfection.

Clonal Expansion

Transfected cells were detached from culture vessels with Accutase-100 (CELLnTEC) 7 days after incubation at 30°C for 3 days. Cells were counted using a hemocytometer and manually seeded using serial dilutions to obtain 1 cell/well of a 96-well plate. Immortalized human keratinocyte clones were co-cultured with 3T3-J2 mouse fibroblasts feeder cells (5–8 × 104 cells/cm2) growth arrested with 4 µg/mL mitomycin C (Roche). Identified edited clones were detached from the surface of 96-well plates and transferred into 6-well plates with mitomycin C-treated feeders for further expansion.

T7 Endonuclease I Assay

Genomic DNA was isolated using phenol/chloroform extraction 1 week after transfection. The TALEN target site was PCR-amplified using Phusion polymerase (New England Biolabs) and analyzed by 0.8% agarose gel electrophoresis. The T7E1 assay for NKc21, EB11, EB21, and primary keratinocytes was performed as follows: forward primer 5'-CAGCTCCCAAACCTGACATGTG-3' and reverse primer 5'-CCCTCCAGCTGGCAGTCTA-3' using 98°C for 30 s, 35 cycles of 98°C for 10 s, 65°C for 20 s, 72°C for 15 s, and 72°C for 7 min. Bands of corresponding size were extracted and purified using the GeneJet gel extraction kit (Thermo Scientific). The DNA fragments were then subjected to digestion with T7 endonuclease I (T7EI) (New England Biolabs). For the T7EI assay, 200 ng of DNA was denatured at 95°C for 5 min, slowly cooled down to room temperature to allow the formation of heteroduplex DNA, treated with 5 U of T7EI for 15 min at 37°C, and analyzed by 1.5% agarose gel electrophoresis. Quantification of gene disruption efficiency (as shown in Figure 2) was carried out according to Guschin et al.53

Genotyping

After achieving a sustainable size (>500 cells per clone) 10–200 cells of each clone were scraped off and collected using a 10-µL micro-pipette tip. TALEN target sites were analyzed by releasing genomic DNA from the collected cells using a Direct PCR kit (Thermo Scientific) according to the manufacturer’s instructions, which was then amplified using Phusion polymerase (New England Biolabs). Direct PCR of EB11 clones was performed as follows: forward primer 5'-CACCTCCCAAACCTGACATGTG-3' and reverse primer 5'-CCCTCCAGCTGGCAGTCTA-3' using 98°C for 30 s; 35 cycles of 98°C for 10 s, 65°C for 20 s, and 72°C for 15 s; and 72°C for 7 min. Direct PCR of EB21 clones was performed as follows: forward primer 5'-CAGCTCCCAAACCTGACATGTG-3' and reverse primer 5'-ACTTTGGTGTCAGCAGATTG-3' using 98°C for 30 s; 35 cycles of 98°C for 10 s, 64°C for 20 s, and 72°C for 30 s; and 72°C for 7 min. PCR products of clones were analyzed by 0.8% agarose gel electrophoresis, and bands of the correct size were isolated, purified using GeneJET gel extraction kit (Thermo Scientific), and sequenced. Sanger sequencing was performed by Source Bioscience.

Allele-Specific PCR

WT and mutant alleles were analyzed by PCR amplification and sequencing using reverse primers binding to the point mutation or
WT sequence. Alleles of EB11 clones were amplified using Phusion polymerase (New England Biolabs). Alleles of EB21 clones were amplified using Taq polymerase (Thermo Scientific). For EB11, the following were used: forward WT primer 5'-TGCTTCAACAGG AACAAGCC-3', forward mutant primer 5'-GCTCCAGGAAACA AGCC-3', reverse WT primer 5'-ACCTGATATTACGGCCT-3', and reverse mutant primer 5'-ACCTGATATTACGGCCT-3'. For EB21, the following were used: forward primer 5'-GCTCCACAGAAGAACGGCC-3', reverse WT primer 5'-CTGCTCAAGCGAACGAC-3', and reverse mutant primer 5'-CTGCTCAAGCGAACGAC-3'. PCR products of alleles were analyzed by 0.8% agarose gel electrophoresis, and bands of the correct size were isolated, purified using the GeneJET gel extraction kit (Thermo Scientific), and sequenced.

Western Blotting
Total protein was extracted from cell cultures and electrophoretically separated and blotted as described previously.54 For antigen detection, rabbit anti-K5 (AF138, Covance), 1:2,000, was used, and Coomassie staining was used to confirm equal loading. Horseradish peroxidase-conjugated species-specific secondary antibodies (Dianova) were used, and immunodetection was performed using Super Signal West Pico and Dura substrates (Pierce, Thermo Scientific), and the data are presented as mean value ± SEM.

Stress Assay
Cells were cultured for 4 days to reach 80%–90% confluence as described below and subjected to stress. Cells were subjected to thermal stress as described previously.5 Briefly, cells were transferred to a closed 43°C water bath for 30 min. Cells were then allowed to recover at 37°C for 15 min, followed by washing with PBS (without Ca2+ and Mg2+), fixation, and immunocytochemistry. Cells were subjected to hypo-osmotic stress using urea (Sigma-Aldrich) as described previously.5 Brieﬂy, cells were immersed in medium containing 150 mM urea at 37°C for 5 min. Cells were then allowed to recover in normal tissue culture medium for 25 min, followed by washing with PBS, fixation, and immunocytochemistry. To assess the extent of IF abnormalities, the total number of cells and the number of cells containing cytoplasmic keratin aggregates for each condition were quantified by counting four different microscope fields on each coverslip. The results were expressed as a fraction (percentage) of the average total number of cells ± SEM.

Immunofluorescence
Cells were cultured in 12-well plates on glass coverslips in duplicates and fixed at −20°C with methanol for 5 min, followed by acetone for 20 s. K5 was stained with rabbit anti-K5 (AF138, Covance), 1:1,000, and goat anti-rabbit Alexa Flour 488-conjugated secondary antibody (Molecular Probes), 1:400. Nuclei were stained with DAPI. Immunofluorescence imaging was carried out using Zeiss Axio Imager II.

Live-Cell Imaging
Live-cell imaging was carried out using a total internal reflection fluorescence (TIRF) and spinning disk (Nikon) confocal microscope. Cells were cultured in 6-well plates for 4–5 days and subsequently locked in the microscope plate holder and covered with a holder lid 48 hr after transfection with an expression plasmid encoding EGFP-muK10 cDNA. The microscope chamber was maintained at 37°C in a 5% CO2 humidified atmosphere. At the time of stress, the temperature of the plate holder and atmosphere was increased to 43°C for 30 min and quickly cooled to 37°C. Cells were visualized throughout the process up to 4 hr. Photographs were taken every 5 min at four fixed positions for each cell line or clone.

Off-Target Analysis
Potential off-target sites for KRT5 TALENs were identiﬁed using TALE-NT 2.0.36 Sites with the best alignments (lowest scores) and spacer lengths within the optimal spacer ranges (10–15 bp and 20–22 bp) were selected for analysis (Table S1). Genomic DNA was isolated using phenol/chloroform extraction 1 week after transfection. Off-target sites were ampliﬁed using GoTaq polymerase (Promega) with the corresponding primers (Table S2). Bands of corresponding size were extracted and puriﬁed using the GFX PCR DNA and gel band puriﬁcation kit (GE Healthcare). The DNA fragments were then analyzed by T7E1 assay.

Ethics Statement
Written informed patient consent was obtained. Ethical approval was granted by the Newcastle and North Tyneside Research Ethics Committee and was sponsored by the Newcastle upon Tyne Hospitals National Health Service (NHS) Foundation Trust.

SUPPLEMENTAL INFORMATION
Supplemental Information includes eight ﬁgures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2017.06.008.

AUTHOR CONTRIBUTIONS
Conceptualization, J.R.; Investigation, M.A. and U.K.; Resources, J.R.; Writing – Original Draft, M.A.; Writing – Review & Editing, J.R., U.K., T.C., and C.M.; Visualization, M.A.; Supervision, J.R. and C.M.; Funding Acquisition, J.R., T.C., and C.M.

CONFLICTS OF INTEREST
The authors declare no conﬂict of interest.

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