Gene Therapy for Skin Fragility Diseases: The New Generation

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Ex vivo gene therapy is a promising approach to treat devastating skin fragility diseases. March et al. and Takashima et al. report that programmable nucleases—TALENs and CRISPR/Cas9—can safely and efficiently correct genetic defects in cultured adult skin cells, paving the way for broader clinical applications of gene therapies in dermatology.


Current status of therapies for genetic skin disorders

Genetic skin disorders, genodermatoses, which comprise a large group of monogenic diseases often characterized by delayed wound healing, severe skin blistering, scarring, and inflammation, are caused by mutations in selected structural skin proteins (Babu et al., 2015). Many genodermatoses, because of their characteristic features of debilitating pain and disability, may not only adversely affect the quality of life of patients but may also make them susceptible to an early death. This is especially true for two severe autosomal recessive subtypes of epidermolysis bullosa (EB): the Herlitz form of junctional EB (JEB) and recessive dystrophic EB (RDEB). Herlitz JEB is generally caused by mutations in one of the three laminin-332 coding genes, resulting in the loss of functional laminin-332, whereas RDEB is caused by mutations in the COL7A1 gene, encoding type VII collagen (Col7) (Has and Kiritsi, 2015). Less severe genodermatoses, such as epidermolytic ichthyosis (EI) that in most cases results from dominant-negative mutations in either KRT1 or KRT10 (Chamcheu et al., 2011), may not be lethal but may still cause debilitating conditions that significantly impede quality of life. Although the genetic defects that cause EB and EI have been known for more than two decades, there are no cures for these conditions, and current therapy is limited to palliative wound care. March et al. (2019) and Takashima et al. (2019) demonstrate the ability of programmable nucleases to edit mutant genes responsible for EI and RDEB, respectively, providing a proof-of-principle concept for a new class of ex vivo somatic cell–based gene therapy for skin fragility diseases.

To date, the most promising experimental therapies for skin fragility diseases have involved ex vivo gene therapy, including the use of viral vectors to restore expression of Col7 in primary keratinocytes (KCs) in patients with RDEB (Siprashvili et al., 2016) and viral vector–mediated restoration of laminin-332 in primary KCs in patients with JEB (Hirsch et al., 2017). In these two studies, patients were engrafted with epidermal sheets derived from autologous KCs that were gene-corrected with viral vectors, effectively replacing defective epidermis with corrected epidermis. In both trials, initial grafting studies were extremely successful, with restoration of normal skin function at 1 year of follow-up (Hirsch et al., 2017; Siprashvili et al., 2016). Despite its promise, the aforementioned ex vivo gene therapy for JEB and RDEB raises safety concerns related to insertional mutagenesis secondary to viral vectors, which impedes a broader application of this therapy in the clinic.

The discovery of two classes of programmable nucleases—transcription activator–like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) nucleases 9 (CRISPR/Cas9)—has marked a new era in the development of simpler and safer gene therapy for genodermatoses with the potential to eliminate viral vectors (Lehmann et al., 2017). Multiple studies have shown that TALENs and CRISPR/Cas9 can achieve precise gene correction in induced pluripotent stem cells (iPSCs) derived from patients with genetic skin fragility diseases and, more recently, in primary skin cells (reviewed by Vanden Oever et al., 2018). Studies by March et al. (2019) and Takashima et al. (2019) provide further evidence that ex vivo gene editing in primary skin cells using programmable nucleases can be a viable therapeutic option for genodermatoses. March et al. (2019) showed that TALENs could efficiently disrupt the dominant-negative KRT10 allele in EI KCs, ameliorating the disease phenotype, whereas Takashima et al. (2019) used CRISPR/Cas9 to restore the function of Col7 in RDEB fibroblasts by correcting the reading frame of one of the mutant COL7A1 alleles.

Correcting defects in EI and RDEB skin cells using nonhomologous end joining

Both groups exploited the same nonhomologous end joining (NHEJ) pathway to achieve the goals of their distinct therapies. Programmable nucleases introduce double-strand breaks (DSBs) at specific loci of the genome. In the presence of exogenous donor DNA carrying the correct gene sequence and the sequences homologous to the region flanking the DSB, homologous recombination (HR) can replace the defective sequence with the corrected one. HR would be desirable for clinical applications, as it promotes specific gene correction. However, the low efficiency of HR impedes its broad application in gene editing of somatic
cells. In the absence of homologous donor DNA, random mutations are introduced via more robust NHEJ. In many cases, NHEJ leads to a knockout of the allele because of the alteration of the reading frame and introduction of a premature termination codon (PTC), a strategy that was implemented by March et al. (2019). However, occasionally, random mutations introduced by NHEJ can restore the normal reading frame of an allele affected by a frame-shift mutation and correct this allele in the process; this is what allowed Takashima et al. (2019) to correct mutant COL7A1.

**TALEN-mediated gene disruption as a universal gene therapy for EI**

March et al. (2019) attempted to develop a universal strategy to disrupt dominant-negative KRT10 via a single nuclease that would be applicable to multiple patients with EI carrying different KRT10 mutations. To achieve this, the authors identified TALENs that specifically cleaved a region in exon 6 of the KRT10 gene and efficiently disrupted the production of the KRT10 protein from the targeted allele because of a PTC and subsequent activation of the nonsense-mediated decay of mRNA. As a result of the position of the targeted sites, the authors predicted that their TALEN-mediated therapy would be applicable to more than 95.6% of dominant-negative KRT10 mutations. To assess TALEN activity, the authors used primary and immortalized EI KCs and healthy KCs. A single clone expansion of targeted EI KCs identified many clones with the mutant KRT10 knockout. High-efficiency gene disruption (up to 56.8%) and a lack of off-target activity were confirmed by next-generation sequencing. The mutant KRT10 knockout restored intermediate filament stability in EI KC cultures and in an in vivo mouse xenograft model. Importantly, the authors successfully expanded single-cell clones of targeted primary EI KCs with a distinct TALEN-induced mutant KRT10 knockout. Successful clonal expansion of gene-edited EI KCs suggests that the generation of epidermal sheets from these cells is a feasible approach to treat EI. In a clinical scenario, TALEN-mediated therapy for EI would resemble the aforementioned viral-based gene replacement approach that allowed for the correction of the epidermis of a boy with JEB (Hirsch et al., 2017). At the same time, this therapy will not depend on the use of randomly integrating viral vectors to correct the disease-associated defect and therefore may potentially be safer and more reliable (Figure 1a).

**Gene reframing therapy for RDEB using CRISPR/Cas9**

To develop a gene therapy for RDEB, Takashima et al. (2019) took advantage of a different class of programmable nucleases, CRISPR/Cas9, which can be directed by short guide RNAs (gRNAs) to induce precise DSB at endogenous genomic loci. The

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**Figure 1. Novel gene therapy for genodermatoses using programmable nucleases.** (a) Experimental gene therapy for EI will rely on the isolation of KCs from a patient's biopsy sample, followed by TALEN-mediated disruption of dominant-negative KRT10 in these cells and the clonal expansion of corrected KCs to produce epidermal sheets for transplantation onto the same patient. (b) In gene therapy for RDEB, isolated skin fibroblasts will be targeted with CRISPR/Cas9 to restore the reading frame of mutant COL7A1 and then injected back into the same patient either intradermally or intravenously. Because of the high rate of reading frame correction observed in this approach, CRISPR/Cas9-treated fibroblasts will potentially promote a sufficient therapeutic effect even if injected in bulk without single-cell cloning or correctly reframed cells. EI, epidermolytic ichthyosis; KC, keratinocytes; RDEB, recessive dystrophic epidermolysis bullosa.
authors developed a mutation-site-specific CRISPR/Cas9-mediated strategy to restore the reading frame of mutant COL7A1 with the RDEB-causing c.5819delC mutation. Because RDEB is a recessive disorder, the allele inactivation strategy suitable for EI is not applicable. Instead, the expression of functional Col7 from at least one COL7A1 allele should be restored. The c.5819delC in COL7A1 is a prevalent frameshift mutation. Therefore, multiple patients with RDEB who share this mutation may benefit if the reading frame of the COL7A1 allele carrying this mutation is restored.

The authors initially used HEK293 cells transfected with a partial COL7A1 sequence containing the c.5819delC mutation to prescreen for the most efficient gRNA. Subsequent Sanger sequencing revealed a high specificity of the designed CRISPR/Cas9-mediated strategy for the sequence containing the mutation, but not the normal sequence (77.2% vs 4.0% targeting efficiency, respectively), with a high rate of reading frame correction (65%). The authors then used primary and immortalized RDEB fibroblasts with the compound heterozygous mutations c.5819delC and c.1474_1481del8 to evaluate the efficiency of their reframing strategy. Multiple clones with correct reframing of the mutant c.5819delC COL7A1 allele were found. No modifications of the other COL7A1 allele or off-target cleavage events were detected, indicating the high specificity of this approach for the mutation site. The functionality of Col7 produced from reframed COL7A1 was confirmed at both molecular and functional levels. The authors also performed an in vivo analysis by injecting reframed RDEB fibroblasts intradermally into immunodeficient mice, followed by assessing the deposition of human Col7 in the dermal-epidermal junction of mouse skin. The corrected primary RDEB fibroblasts were also injected into skin that was collected from a Col7 knockout mouse and subsequently engrafted onto immunodeficient mice. Correct deposition of human Col7 was detected, and anchoring fibrils were formed at the dermal-epidermal junction in this in vivo model. Given the high rate of reading frame correction, the authors speculated that their strategy might allow for intradermal or intravenous injections of CRISPR/Cas9-treated fibroblasts into affected patients in bulk without single-cell cloning of correctly reframed cells, thus shortening the duration of the therapy (Figure 1b). Although technically feasible, an approach where corrected and uncorrected cells are injected in bulk without single-cell expansion of corrected clones may be risky. Single-cell cloning of correctly reframed RDEB fibroblasts would minimize the risk of unwanted genetic and epigenetic changes that may occur during genetic correction. It remains to be determined if RDEB KCs can be reframed using the CRISPR/Cas9 strategy designed by Takashima et al. (2019). Reframing RDEB KCs followed by their single-cell expansion and the generation of epidermal sheets for transplantation may be a better strategy to treat EB because of the high proliferative capacity of epidermal cells. In fact, in the report describing the successful viral-based gene replacement therapy for JEB, a relatively small number of KCs were expanded to a sufficient number to replace the entire epidermis of a patient with JEB (Hirsch et al., 2017).

Concluding remarks
The studies by March et al. (2019) and Takashima et al. (2019) represent the latest attempts to develop gene therapy for skin fragility diseases. Despite differences in their gene editing strategies, both groups show that programmable nucleases can be used for efficient and safe gene correction of skin cells without the use of integrating viral vectors, selection strategies, or more complex iPSC technology. One question remains: what is the best programmable nuclease for gene therapy, CRISPR/Cas9 or TALEN? Both nucleases have their advantages and disadvantages (reviewed by Nerys-Junior et al., 2018). CRISPR/Cas9 has a major limitation, as it requires the presence of a three-base protospacer adjacent motif (PAM) sequence (NGG preferred, where “N” can be any nucleotide base followed by two guanine nucleobases) immediately after the gRNA target sequence on the DNA. TALENs, which initially required target sequences with a preceding 5’ thymine, can now be designed to bind to and cleave virtually any sequence regardless of the presence of thymine. In fact, March et al. (2019) focused on TALENs because this strategy allowed the authors to target a short region within KRT10, which would have been difficult to cleave with CRISPR/Cas9 because of the lack of an appropriate PAM sequence. On the other hand, TALENs are usually harder to produce and therefore may be more expensive for clinical manufacturing than CRISPR/Cas9. In many cases, CRISPR/Cas9 also shows a higher cleavage efficiency than TALENs. To summarize, CRISPR and TALENs are both potentially suitable for gene therapy, and the decision to pick one nuclease over the other depends on several parameters, such as cell type, gene editing strategy, and genomic loci to be targeted. Although additional studies are needed to improve the safety and efficacy of TALENs and CRISPR/Cas9, the studies by March et al. (2019) and Takashima et al. (2019) clearly mark the beginning of a new era in developing safer and more effective gene therapies for genodermatoses.

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CONFLICT OF INTEREST
The author states no conflict of interest.

REFERENCES

