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Gene editing toward the use of autologous therapies in recessive dystrophic epidermolysis bullosa

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Abstract

Recessive dystrophic epidermolysis bullosa (RDEB) is a disease caused by mutations in the *COL7A1* gene that result in absent or dysfunctional type VII collagen protein production. Clinically, RDEB manifests as early and severe chronic cutaneous blistering, damage to internal epithelium, an elevated risk for squamous cell carcinoma and an overall reduced life expectancy. Recent localized and systemic treatments have shown promise for lessening the disease severity in RDEB, but the concept of *ex vivo* therapy would allow a patient's own cells to be engineered to express functional type VII collagen. Here we review gene delivery and editing platforms, and their application toward the development of nextgeneration treatments designed to correct the causative genetic defects of RDEB.

INTRODUCTION

Epidermolysis bullosa (EB) represents a heterogeneous group of diseases characterized by errors in genes that encode the structural components of the skin. Clinical manifestations of EB primarily involve chronic blistering and poor wound healing of cutaneous and mucosal surfaces, with the severity of disease dictated by the specific underlying genetic mutation and degree of protein dysfunction. While there are more than 20 subtypes of EB, one of the most severe forms is generalized severe recessive dystrophic EB (RDEB).[1] RDEB results from biallelic loss-of-function mutations within the collagen type VII gene (*COL7A1*) that lead to absent or deficient production of normal collagen type VII protein (C7). In healthy skin, keratinocytes and dermal fibroblasts secrete procollagen VII, which is processed into C7 and assembled into anchoring fibrils (AFs).[2] AFs provide the main structural connection between the papillary dermis and epidermal basement membrane zone (BMZ), thus providing a "biologic Velcro" at the dermal-epidermal junction (DEJ).[3] In patients with RDEB, dermal-epidermal integrity is compromised by the diminished presence of functional

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C7. This fundamental defect produces the characteristic RDEB clinical presentation of chronic and severe skin blistering; mitten deformities of the hands and feet; corneal erosions; oral, esophageal, and anal strictures; with variable involvement of the heart, kidneys, and bones.[4, 5]

EPIDEMIOLOGY, GENETICS, and HISTORICAL APPROACH TO TREATMENT

Affecting approximately 1/1,000,000 newborns in the United States, RDEB is often evident at birth and is associated with a life expectancy of 30 years in severe cases, with milder phenotypes exhibiting a median survival of 55–65 years.[6, 7] In addition to severe pain and frequent failure to thrive, patients are at exceptionally high risk (≈80%) for developing aggressive forms of squamous cell carcinoma (SCC), owing to the chronic remodeling and increased cell proliferation occurring at sites of mucocutaneous blistering.[4]

Genetics of the COL7A1 Locus and Processing of C7 Protein

The human *COL7A1* gene, located on chromosome 3p21, encompasses 32 kb of genomic DNA and contains 118 exons. The resultant mRNA transcript is 8.9 kb in length and is translated into a proα1 (VII) polypeptide composed of 2944 amino acids. Exons 1–28 of the mRNA transcript represent the N-terminal “non-collagenous” (NC-1) domain; exons 112–118 represent the C-terminal “non-collagenous” (NC-2) domain; while the intervening central “collagenous” triple helical domain contains varying stretches of bases coding for Gly-X-Y repeat sequences with disrupting “non-collagenous” regions throughout. Thus, each proα1 (VII) chain contains a central “collagenous” triple helical domain flanked by the NC-1 and NC-2 domains. Three proα1 (VII) chains polymerize to form a procollagen VII homotrimer, the secretory product of C7-producing cells. Procollagen VII homotrimers undergo extracellular processing to yield the functional C7 protein product, which assembles with another C7 molecule at the carboxy-terminal region to form anti-parallel dimers with NC-1 domains facing opposing ends of the dimer. The NC-1 domains within each dimer are involved in forming adhesive bonds with extracellular matrix proteins (e.g., collagen IV) that help stabilize AFs to the papillary dermis and BMZ of the epidermis. The triple helical regions in each dimer form cross-bonds with neighboring homotrimers, and the NC-2 domains are proteolytically cleaved after dimerization and prior to AF formation.[8–10] Anti-parallel dimers then assemble to form AFs, which play a crucial role in the structural integrity of the DEJ.

Patients with RDEB are often compound heterozygotes, with biallelic premature termination codons (PTCs) being prevalent in the generalized severe subtype of RDEB (previously called Hallopeau-Siemens RDEB).[8, 11–13] PTCs result from nonsense, frameshift, or splice-site mutations that cause truncation of the mRNA transcript and a non-functional protein that precludes normal AF formation. Patients with less severe subtypes (e.g., generalized intermediate RDEB) typically have a PTC on one allele and a non-PTC mutation (e.g., missense, deletion/insertion) on the other allele, which results in a partially functional polypeptide.

Clinical management of RDEB has classically involved measures aimed at palliative wound care and pain management. More recently, experimental therapies have been developed seeking to provide functional C7 protein at sites of involvement. The C7 product has been delivered either directly as a recombinant protein, or via introduction of allogeneic donor cells that synthesize the protein *in vivo*. Both approaches have shown some benefits, albeit non-uniform ones, in pre-clinical models and phase I human studies. The inability to achieve a complete therapeutic response provides impetus for developing more effective treatment options. These include improvements to existing recombinant protein and allogeneic cellular therapies, as well as genetically-corrected autologous cellular platforms for C7 protein delivery.

Recombinant Protein Therapies

The use of human recombinant C7 protein as a potential treatment for RDEB patients was first described in 2004 with the demonstration that intradermal injections of the protein could localize to the BMZ and produce functional anchoring fibrils in murine and human RBEB skin models.[14] Owing to the relatively long physiologic half-life of the molecule, C7 was shown to be present at the DEJ throughout the three-month observation period. Subsequent studies extended these findings into a *Col7a1*$^{-/-}$ murine model, showing similar benefit.[15] Although these mice developed antibodies against the C7 protein, this did not preclude formation of anchoring fibrils and improvement of the disease phenotype. Given the presumed advantage of a systemic intravascular delivery method—which could theoretically provide recombinant C7 protein to all mucocutaneous lesions rather than being restricted to injection sites—this same group provided evidence in 2013 that intravenous infusions of recombinant C7 could home to the DEJ and form anchoring fibrils in an RDEB skin model.[16] While these pre-clinical studies hold translational application and provide proof-of-concept for recombinant protein-based therapies, the likely need for repeated injections, with the associated costs and the possibility of anti-C7 antibody formation, may limit the efficacy of this approach.[17]

Allogeneic Cellular Therapies

The principle behind allogeneic cellular therapies in RDEB is that by introducing a wild-type donor cell capable of producing normal C7 protein, improved DEJ stability can be achieved by donor cell contribution to AFs. Wong *et al*. found that in patients receiving intradermal allogeneic fibroblast injections, increased C7 could be found at the DEJ for up to three months, but the AFs were not of normal morphology, presumably due to the limited amount of C7 deposited before rejection of donor cells.[18] In a later study of 11 RDEB patients receiving localized intradermal allogeneic fibroblast injections, wound healing was improved for the first 28 days but not thereafter.[19] As an alternative to fibroblasts, mesenchymal stem cells (MSCs) have also been used as an intradermal allogeneic therapy. MSCs were shown to improve wound healing and deposit normal C7 at the DEJ in two patients. The clinical benefit was seen for four months, indicating that MSCs could contribute *de novo* C7.[20] These results were supported by later studies that found MSCs capable of restoring partial DEJ function in RDEB skin.[21] These findings are promising given the generalized immune-modulatory properties of MSCs, which have been shown to promote wound healing and decrease fibrosis.[22, 23] Similar to recombinant protein, the

short-lived persistence of allogeneic cells would mandate repeated delivery of the C7 vehicle, making it desirable to identify a population of cells that persist long term.

To that end, hematopoietic progenitor cells have been shown to have beneficial effects in injury models such as myocardial infarction, where they are able to travel to sites of injury to improve functional outcomes.[24, 25] To determine whether this procedure would be beneficial in RDEB, pre-clinical studies in RDEB mice that underwent hematopoietic stem cell transplantation (HCT) were performed and increased survival rates were observed.[26] These studies served as proof-of-concept for the first human phase I trial of HCT in RDEB patients, in which amelioration of many of the external features of the disease phenotype was documented.[27] Due to conditioning regimen and transplant-related side effects, the trial has since been reconfigured to employ a reduced intensity conditioning regimen and to determine whether functional outcomes can be improved using MSCs.[28] This study represents a strategy for potentially lifelong therapy from engrafted donor stem cells, which could obviate the need for repeated injections. However, the challenges associated with HCT provide an impetus for the development of personalized *ex vivo*-based therapies. The ability to modify a patient's specific *COL7A1* mutation to wild-type status would allow for the correction, expansion, and delivery of autologous cells aimed at treating the whole patient, while also avoiding the immunological complications of allogeneic therapies.

IDEAL CELL TYPE for GENETIC THERAPY in RDEB

Due to the relative ease of procurement and of cell line derivation *in vitro*, fibroblasts and keratinocytes have been the cell types most frequently used in studies of gene therapy for RDEB. In terms of functional significance, fibroblasts have been shown to contribute more C7 production at the DEJ relative to keratinocytes.[18, 29] However, the significant limitation with both cell types is that they have finite lifespans *in vivo*. Fibroblasts, while considered to be more robust and to exhibit less growth arrest and differentiation compared to keratinocytes,[30] senesce rapidly *in vivo*.[31] Due to rapid turnover, injected or grafted keratinocytes also fail to persist long term in dermal-epidermal tissue.[32, 33]

Thus the epidermal stem cell emerges as an attractive cell type for autologous gene correction and has been shown to be amenable to such techniques.[29, 34, 35] Given the limited quantity of epidermal stem cells available for procurement *in vivo*, a corresponding approach involves nuclear re-programming of adult cells into undifferentiated induced pluripotent stem cells (iPSCs). iPSCs resemble embryonic stem cells in their capacity to differentiate into the hundreds of different specialized cell types found in the complex tissues of mammalian organs. This broad differentiation profile and their use as disease modeling tools makes them uniquely suited for RDEB regenerative therapies. Specifically, the derivation of iPSCs from RDEB fibroblasts and keratinocytes, and the genetic correction of these cells—along with rigorous quality assurance and controls to remove the risk of reprogramming-based mutation accumulation—hold great promise for RDEB therapy.[32, 36–40]

GENE THERAPY and EDITING in RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

Beginning in the early 2000s, studies using non-viral, viral-, and phage-mediated gene therapy vectors have been employed for ectopic expression of C7 in patient-derived RDEB cells. More recently, the explosion of readily available and highly specific gene-editing platforms has made the goal of individualized patient-centric therapies an achievable one.

Plasmid DNA Transfection

The large size of the *COL7A1* gene (32 kb) and mRNA/cDNA molecules (≈9 kb) can be prohibitive for its use in size-restricted vectors. Because plasmid-based therapies are largely unrestricted in regards to cargo capacity, they have been used to deliver full-length *COL7A1* cDNA directly into rat epidermis via local injections. However, only a small population of keratinocytes were found to express C7 and the duration of expression was limited to one week.[41] Mecklenbeck *et al*. microinjected a P1-derived artificial chromosome (PAC) containing the entire 32 kb human *COL7A1* locus and its neighboring genomic sequences into immortalized, patient-derived, RDEB keratinocytes and demonstrated sustained biosynthesis and secretion of procollagen VII for up to one year.[42] While these results provided evidence that genomic DNA constructs could restore C7 production, the PAC construct often integrated at multiple chromosomal locations. Thus, while establishing the practicality of *COL7A1* genomic DNA transfection, this study also demonstrated the need for methods with more efficient delivery techniques and more precise control of integration events.

Viral- and Phage-Mediated Transduction

Viral vectors have historically been the delivery method of choice for studies of RDEB gene therapy. Retroviral (RV) and lentiviral (LV) vectors are capable of integrating into the host genome to allow for stable and long-term gene expression, but the limited cargo capacity of these vectors can greatly influence titers and gene transfer rates. In 2000, Chen *et al*. used an RV vector with a truncated *COL7A1* "mini-gene" to accommodate these size limitations. They were able to demonstrate that immortalized RDEB keratinocytes could be transduced to express a mini-C7 protein product that improved the cellular RDEB phenotype via normalizations in cell motility, cell adhesion, and proliferation rates.[43] In 2002, this same group used a self-inactivating LV-based vector to deliver a full-length *COL7A1* cDNA sequence into patient-derived RDEB keratinocytes and fibroblasts.[44] They reported >95% transduction efficiency and demonstrated persistent synthesis and secretion of normal C7 throughout the five month *in vitro* observation period. During the two-month study period, these corrected cells were able to produce normal anchoring fibrils when grafted onto immune-deficient mice. In 2003, Baldeschi *et al*. reported the first use of an RV vector to transduce a full-length *COL7A1* cDNA sequence into RDEB keratinocytes, while also providing evidence that epidermal stem cells can be successfully corrected.[34] Woodley *et al*.,[45] using a similar LV-mediated system as Chen *et al*.,[44] performed intradermal injections of corrected patient-derived RDEB fibroblasts to restore C7 at the DEJ for four months in an RDEB skin model. This group later demonstrated that direct intradermal injections of an LV

vector containing *COL7A1* cDNA could produce stable expression of human C7 in fibroblasts and endothelial cells for at least three months in a murine model.[46]

The use of long terminal repeats (LTRs) or exogenous promoters in self-inactivating (SIN) LVs and DNA-based expression platforms can result in sustained gene expression that is not subject to the regulatory mechanisms of the cell. Given the potential for C7 to contribute to fibrosis, unchecked gene expression may be detrimental. Furthermore, both RVs and LVs show a bias toward integrating into transcriptionally active regions of the genome.[47] These factors raise significant concerns for adverse events related to insertional mutagenesis, which have been documented in previous RV gene therapy trials that resulted in vector-induced leukemogenesis.[48, 49] Despite these earlier setbacks to viral-mediated gene therapy, more recent improvements in SIN LV design have shown their benefit in pilot studies for monogenic diseases without the occurrence of overt insertional mutagenic-induced phenotypes.[50, 51]

As an alternative to viral-mediated transduction, Ortiz-Urda *et al*. used a phage-mediated platform to deliver *COL7A1* cDNA into patient-derived RDEB primary epidermal progenitor cells.[35] The authors utilized a phiC31 phage integrase, which can integrate large (up to 10 kb) DNA sequences containing a specific 285 bp *attB* sequence into genomic "pseudo-*attP* sites." Most epidermal progenitor clones had *COL7A1* integration at predictable locations based on known pseudo-*attP* sites. Random integrations were also seen, but none were within gene-encoding regions. This technique had relatively lower transfection efficiency rates (roughly 45% at two days) than previously reported viral transduction methods, but through culture expansion and selection of C7-producing cells, the authors were able to achieve a >99% success rate after a ten-day selection period. Contribution of C7 production by epidermal progenitor cells was suggested by persistent expression for 14 weeks, thus spanning multiple turnover cycles of keratinocytes.[52] This group published a follow-up study in which the same phiC31 phage integrase platform was used to correct patient-derived RDEB fibroblasts. Corrected fibroblasts were then injected into an RDEB skin model and were shown to restore C7 at the DEJ.[30] However, the requirement of the phiC31 integrase gene, the lack of responsiveness to endogenous gene regulation, and the potential for random insertional mutagenesis may be limiting for phage therapy.

In contrast to integration-based viral vectors, adenoviral vectors exist as extra-chromosomal elements that can accommodate large cargo sizes (≈35 kb). This platform would make it more amenable to transfer *COL7A1* cDNA alongside natural promoter elements to create an environment responsive to localized cues for gene regulation. However, the immune-activating properties of this platform and its existence as an extra-chromosomal species, which restricts its expression to the lifespan of the transduced cell type, are both limiting factors to the widespread use of this platform.[53–55] Unlike adenoviral vectors, "adeno-associated" viral (AAV) vectors are capable of integrating into the host genome. While unable to accommodate large vector insertions, AAVs are considered much lower risk relative to other viral platforms (Table 1).[56]

Gene Editing

The limitations of localized cell and protein therapies, HCT, and gene therapy vectors have made geneediting nucleases an attractive option for RDEB therapy. These reagents can be rationally designed and engineered to mediate a break in the DNA strand at a user-defined locus resulting in a 100- to 1000-fold rate increase in homologous recombination.[57, 58] Candidate platforms for gene editing include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system. ZFNs, TALENs, and CRISPR/Cas9 function similarly in that they all bind a user-defined DNA sequence and mediate a break in one or both of the DNA strands. However, their architecture and engineering processes differ greatly (Table 2).

ZFNs are heterodimeric DNA-binding proteins fused to a FokI nuclease domain and co-localize to a target site to mediate a DNA break. Each of the individual finger motifs within a ZFN array contacts 3 bp of DNA. These motifs are derived from the Zif268 zinc finger domain but are engineered for site specificity using highly complex and labor-intensive methodologies.[59] ZFN constructs, typically containing between three- and six-finger units, are generated as heterodimeric arrays separated from one another by a 5–7 bp ‘spacer’ region in which the DNA break occurs.[60, 61] The relatively small size of ZFN arrays makes them highly flexible for use for many gene delivery platforms; however, the requisite purchase and/or industry affiliation, or the acquisition of specialized starting materials and generation methodologies, could restrict their widespread use.

Similar to ZFNs, TALENs function as dimeric proteins and are tethered to the FokI nuclease; however, the spacer region in which DNA cleavage occurs is larger.[62, 63] TALEs are comprised of repeating elements that each contact a single base of DNA. This recognition is governed by amino acids at the 12 and 13 positions, termed repeat variable di-residues (RVDs). Thus, a 1:1 relationship exists between the RVD in a repeat sequence and the base it recognizes as part of a simple code (HD=C, NN/NK=G, NI=A, NG=T) that allows sequence-specific TALEs to be generated.[64–66] To determine whether TALENs could be applicable toward RDEB gene correction, our group engineered proteins recognizing sequences proximal to a homozygous PTC in exon 14 of the *COL7A1* gene. The TALENs and an exogenous plasmid donor sequence spanning exons 12–15 were introduced into patient-derived primary fibroblasts. Homologydirected repair was observed in 44% (8/18) of the clones where the TALEN system was delivered as plasmid DNA, and in 12.5% (2/16) of cases where it was delivered as mRNA. Fifty percent (50%) of the clones showing evidence of homology-directed repair via the plasmid DNA delivery system were observed to have a corrected mutation, while 25% were found to incorporate the entire full-length donor-derived template. Corrected fibroblasts were reprogrammed to pluripotency and were able to form skin-like structures containing localized C7 at the DEJ in an *in vivo* teratoma differentiation assay.[38]

The most recently described gene editing platform is the highly user-friendly CRISPR/Cas9 system. This is a bacterial adaptive immune system that is mobilized in response to phage infection and has been repurposed for mammalian cell use.[67] It functions as an RNA-guided

endonuclease (RGEN) comprised of a small guide RNA (gRNA) transcript that associates with the Cas9 protein, which contains two nuclease domains that cleave the individual strands of DNA.[68] The most commonly used CRISPR/Cas system is derived from *Streptococcus pyogenes* and employs site-specific cleavage via Watson-Crick base pairing of the Cas9:gRNA to a target sequence proximal to a protospacer adjacent motif (PAM), which is essential for specificity of Cas9 binding.[67] The PAM sequence is heterogeneous between the different CRISPR/Cas systems, with the *S. pyogenes* system utilizing an -NGG motif and a full target recognition sequence of $G(N_{19})$-NGG.[69]

The utilization of ZFNs, TALENs, and CRISPR/Cas systems will ultimately be driven by availability, and gene targeting frequencies and specificities. For instance, the targeting capacities of these three systems within the genome are fundamentally different: ≈1:500 bp for ZFNs and ≈1:35 bp for TALENs, while *S. pyogenes* CRISPR/Cas systems can target ≈40% of human exons.[70–72] These considerations, along with the potential for promiscuous nuclease activity at sites of similar sequence homology, all factor into the utility of gene editing proteins for translational use. For example, reagents generated for specificity at the *CCR5* locus have shown off-target (OT) effects at the *CCR2* locus, which shares significant homology with *CCR5*.[73–75] These findings highlight the importance of rigorous target site selection analyses that emphasize sequence heterogeneity. Such design strategies can be combined with optimized architectures and ultrasensitive OT site identification methodologies to support translational use of these systems.[37, 76–80]

Nuclease-free targeting of the *COL7A1* locus has also been demonstrated by Sebastiano *et al*.[32] Two patient-derived RDEB cell lines with mutations in exons 2 and 3 of the *COL7A1* locus were targeted by an AAV-mediated system that spanned six exons and contained 1.4 kb targeting arms on either side of a central puromycin selection cassette. When compared to a larger, more conventional targeting vector with 8.8 kb and 4.4 kb arms spanning 31 exons, the AAV-mediated system produced greater recombination frequencies [56% (17/30 clones) and 16% (1/6 clones) for the two patient lines], with targeted clones being successfully corrected in 6% (1/17) and 100% (1/1) of these recombination events. G-banding analyses confirmed normal karyotypes post correction, while Southern blotting and Sanger sequencing confirmed an absence of random integration events within the *COL7A1* locus. Importantly, the corrected cell types in these experiments were iPSCs derived from RDEB keratinocytes and fibroblasts. The corrected iPSCs were then transformed into corrected keratinocytes, which were used to create autologous epithelial grafts with normal C7 composition. Furthermore, by genotyping 13 known SCC-predisposing genes in each lineage, the researchers were able to create a screening system whereby corrected cells exhibiting the lowest mutational burden could be applied to the autologous grafts. Toward realizing the full potential of gene editing, future strategies may maximize rates of homology-directed repair by using nucleases to generate DNA breaks in the context of recombinogenic AAV-based donor molecules.[81]

Interestingly, certain RDEB patients have been shown to harbor cell populations containing normal *COL7A1* alleles within areas of healthy skin in a phenomenon referred to as “revertant mosaicism.”[40, 82–84] Such cell populations may afford the opportunity to bypass

the gene therapy/editing procedures by isolating and expanding these subpopulations for redelivery to the patient.

THERAPEUTIC DELIVERY of CORRECTED AUTOLOGOUS CELLS for RDEB

Future treatment modalities will most likely be combinatorial in nature. For localized therapy, combining gene editing/therapy with tissue engineering of three-dimensional skin grafts represents a novel methodology for providing normalized C7 at the DEJ. By grafting this tissue onto sites of involvement supportive local effects can be achieved. However, the financial and temporal cost of repeated grafting procedures, the fragility of epidermal tissue created *ex vivo*, and the excision of wounded tissue prior to grafting with potential for subsequent scarring are all potential limitations of this localized approach.[29] The combinatorial concept can be expanded to include merging localized and systemic platforms using gene corrected hematopoietic progenitors. The ability to employ gene-editing nucleases in HSCs has recently been described by Genovese et al in severe combined immunodeficiency and represents a strategy for ex vivo therapies designed to mitigate transplant related toxicity.

Next generation therapies—whether local, systemic, or both, whether for individual wounds or for a patient with generalized severe RDEB—that are based on gene editing of *COL7A1* mutations in fibroblasts, keratinocytes, MSCs, or HSCs, involve a complex multistep process. To be useful in the clinic, these steps—including design, production, and delivery of molecular tools; preparation and expansion of relevant cells; and local or systemic grafting of the gene-edited cells—must be validated and simplified. The common theme in successful examples of translation is a disappearance of the distance between the bench and the bedside with a cognizance that to make such research clinically meaningful: the technology has to be robust, scalable, suitable for regulatory approval, and achieve more complete therapeutic outcomes.

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Abbreviations

RDEB	recessive dystrophic epidermolysis bullosa
C7	type VII collagen protein
AF	anchoring fibril
BMZ	basement membrane zone
DEJ	dermal-epidermal junction
SCC	squamous cell carcinoma

PTCs	premature termination codons
HCT	hematopoietic cell transplantation
MSC	mesenchymal stem cell
iPSC	induced pluripotent stem cell
PAC	P1-derived artificial chromosome
RV	retrovirus
LV	lentivirus
LTRs	long terminal repeats
SIN	self-inactivating
AAV	adeno-associated virus
TALEN	transcription activator-like effector nuclease
ZFN	zinc-finger nuclease
CRISPR	clustered regularly interspaced short palindromic repeats
RVDs	repeat variable di-residues
RGEN	RNA-guided endonuclease
gRNA	guide RNA
PAM	protospacer adjacent motif
OT	off-target

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Table 1
Comparison of viral- and phage-mediated transfection platforms

The characteristics described here represent historical features of the listed platforms (*cf* review by Anderson[53]). Note that all platforms may exhibit some degree of site-specific targeting depending on homologous overlap with genomic loci. RT = reverse transcriptase; HR = homologous recombination.

Vector Platform	Max Cargo Size	Preferential Cell Cycle Activity	Integration Specificity	Examples in RDEB Therapy
Retrovirus (RV)	≈7–8kb	Dividing cells	Specific via HR or non-specific via RT activity	Chen et al.[43] Baldeschi et al.[34] Goto et al.[29] Murauer et al.[85] Siprashvili et al.[86]
Lentivirus (LV)	≈13kb	Dividing and non-dividing cells	Specific via HR or non-specific via RT activity; ↓ rates of pro-oncogenic insertions vs. RV[87]	Chen et al.[44] Woodley et al.[45] Woodley et al.[46]
Adenovirus	≈35kb	Dividing and non-dividing cells	N/A; exists as extra-chromosomal element	N/A
Adeno-associated virus (AAV)	≈5kb	Dividing and non-dividing cells	Specific via AAVS1 integration or HR, or non-specific via host repair mechanisms[56]	Sebastiano et al.[32]
Φ-C31 phage integrase	≈10kb	Dividing and non-dividing cells	Specific via pseudo-attP integration sites; non-specific via random integration[35]	Ortiz-Urda et al.[35] Ortiz-Urda et al.[30]

Table 2

Comparison of next-generation gene editing techniques

Vector Platform	DNA-binding Domain	Estimated Binding Site Frequency	Nuclease Domain	User-Friendly	Examples in RDEB Therapy
ZFN	Zinc-finger motifs; each recognizes 3 bp sequence	≈1:500 bp	Typically *Fok*I nuclease	+	CompoZr® *COL7A1* ZFN Kit (Sigma – Aldrich®)
TALEN	TALE proteins; RVDs have 1:1 relationship with specific DNA base	≈1:35 bp	Typically *Fok*I nuclease	++	Osborn et al.[37]
CRISPR/Cas	gRNA complementarity proximal to PAM sequence	≈40% of human exons (for *S. pyogenes* system)	Cas nucleases	+++	N/A