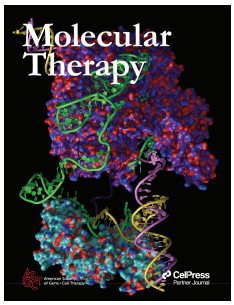

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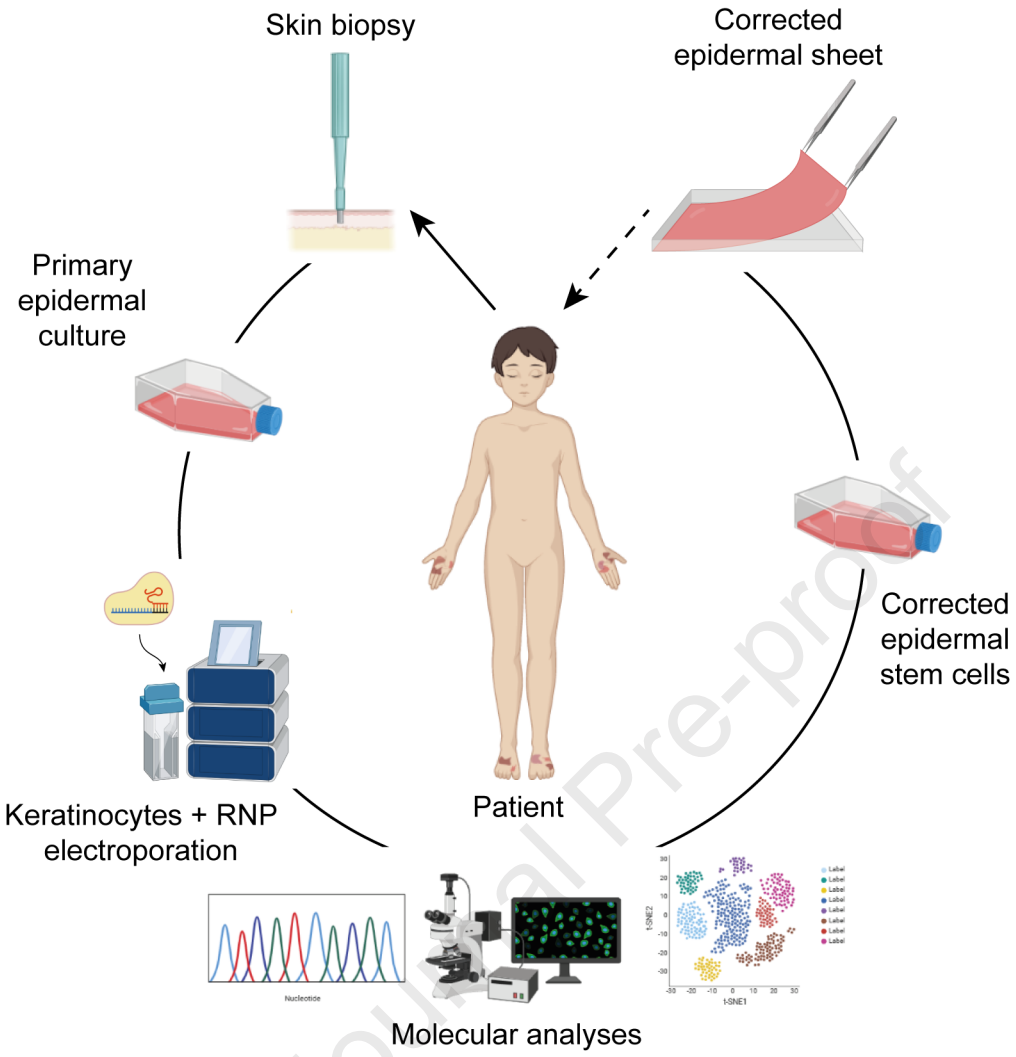

Allele specific CRISPR/Cas9 editing of dominant Epidermolysis Bullosa Simplex in human epidermal stem cells.

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Abstract

Epidermolysis Bullosa Simplex (EBS) is a rare skin disease inherited mostly in an autosomal dominant manner. Patients display a skin fragility that leads to blisters and erosions caused by minor mechanical trauma. EBS phenotypic and genotypic variants are caused by genetic defects in intracellular proteins whose function is to provide the attachment of basal keratinocytes to the basement membrane zone and most of EBS cases display mutations in keratin 5 (*KRT5*) and keratin 14 (*KRT14*) genes. Besides palliative treatments, there is still no long-lasting effective cure to correct the mutant gene and abolish dominant negative effect of the pathogenic protein over its wild-type counterpart. Here, we propose a molecular strategy for EBS01 patient's keratinocytes carrying a monoallelic c.475/495del21 mutation in *KRT14* exon1. Through the CRISPR/Cas9 system we performed a specific cleavage only on the mutant allele and restore a normal cellular phenotype and a correct intermediate filament network, without affecting the epidermal stem cell, referred to as holoclones, which play a crucial role in epidermal regeneration.

Introduction

Inherited EB is a heterogeneous group of rare, autosomal genetic disorders caused by molecular defects within genes encoding structural proteins forming the epidermal–dermal junction. EB is characterized by recurrent blistering and erosions of the skin (and other stratified epithelia) that arise, spontaneously or upon minimal mechanical stress, within the epidermis in EB simplex (EBS), the lamina lucida in Junctional EB (JEB) and beneath the lamina densa in Dystrophic EB (DEB). EBS is the most common EB form, with a prevalence of 1/30000 - 1/50000.[1,2] Its clinical manifestations are usually less severe than those of JEB and DEB, which can be devastating and even early lethal. However, some EBS forms are marked by a severe phenotype and several clinical variants have been identified based on the mutated gene, site of blister formation, anatomical distribution and mode of inheritance.[3–5]

JEB and DEB are mostly recessively inherited, whilst the vast majority of EBS are inherited in a dominant manner. In fact, approximately 75% patients suffering from EBS harbours dominant mutations in *KRT5* and *KRT14*, the genes encoding keratin 5 (K5) and keratin 14 (K14), respectively. K5/K14 pairs form the basal keratinocyte intermediate filaments, which are part of the hemidesmosomal protein complex tethering the epidermal basal layer to the basement membrane and the underlying dermis. Mutant keratins exert a dominant negative effect on the functional keratins encoded by the normal allele, hence perturbing the basal keratinocyte intermediate filament network and leading to intraepidermal blister formation. Thus, whilst JEB and DEB can be tackled by the addition of a corrected copy of the mutated gene in the genome of epidermal stem cells[6–14], a potentially successful combined ex vivo cell and gene therapy of EBS strictly requires editing of the mutated allele.

Here, we outlined an allele specific CRISPR/Cas9 based gene editing approach that is able to disrupt specifically the *KRT14* mutant gene and fully restore functional intermediate filaments in epidermal stem cells, cultivated from an EBS patient carrying a *de novo* monoallelic c.475/495del21 dominant mutation in exon 1 of *KRT14*.

This approach takes advantage of a tailored CRISPR/Cas9 system to induce double strand breaks (DSBs) specifically on the mutant allele, leading to non-homologous end joining (NHEJ) repairing process. These rearrangements are likely to generate frameshift mutations resulting in both pathogenic allele expression abolishment and phenotypic and mechanical stress resilience restoration. Besides the remarkable efficacy of this approach, we also demonstrate the correction of the epithelial stem cells compartment, which is mandatory for the long-term skin regeneration. This highly effective and safe gene editing strategy would therefore enable a translation to clinical application for the treatment of other dominant form of EB.

Results

Novel monoallelic *KRT14* deletion causing a dominant form of Epidermolysis Bullosa Simplex

An 8-year-old EBS patient (referred to as EBS01) suffered from a *de novo* heterozygous dominant mutation (c.475/495del21) within exon 1 of *KRT14*. The patient developed bullous skin lesions few months after birth and currently presents blisters in the palmoplantar region causing postural and ambulation problems. No other cases of EB were known among his relatives. Besides the palliative care and a regular multidisciplinary follow-up, no resolutive treatment is available for this patient (Fig. 1A).

The EBS01 variant results in the deletion of seven in-frame amino acids, leading to a shorter K14 protein. The AlphaFold2[15] suite for protein structure prediction and modeling was used to predict the 3D-structure of the shorter K14 (Fig. 1B). The *c.475/495del21* variant affects the protein structure resulting in the absence of an extended loop, Linker L1, encompassing residues 159–165 (Fig. 1B).[16] The L1 structural motif of K14, whose function is still poorly characterized, is predicted to assume a highly flexible, non-helical β-turn, with the pivotal function of connecting coil 1A with 1B (Fig. 1C).[17] The importance of the L1 motif is also reflected by its high evolutionary conservation in keratins.[18] Indeed, mutations affecting the linker regions of intermediate filaments have been previously observed and related to severe cases of inherited skin blistering diseases, highlighting the unexpected sensitivity of these regions to structural alterations.[17]

Efficient and precise *KRT14* allele-specific editing in EBS primary keratinocytes

The EBS01 genetic variation enables the mutant K14 to exert a dominant negative effect over its wild-type counterpart expressed by the other allele. Thus, a tailored CRISPR/Cas9 system was employed to target the monoallelic *de novo* mutation (*c.475/495del21*) within exon 1 of *KRT14* to induce a deleterious double strand break on the mutant allele and promote NHEJ, inducing specific

disruption of the mutant *KRT14* open reading frame. We designed a sgRNA tailored to specifically target only the mutant *KRT14* allele and employed the SpCas9 to specifically recognize the "NGG" PAM present near the deletion site. For the desired specificity, the 19 nucleotides long guide RNA was sketched straddling both the terminal sides of the 21 base pair deletion and directly flanking the "AGG" PAM sequence within its 3' end (Fig. 2A).

To first assess the ability of the designed CRISPR/Cas9 system to specifically abolish the expression of the *KRT14* mutant allele, preliminary experiments employed a lentiviral vector to deliver the gene editing machinery to EBS01-derived primary keratinocytes.

Strikingly, the gene editing machinery was able to disrupt the expression of the mutant *KRT14* allele with an efficiency up to 94%, without affecting the wild-type allele (Fig. S1A). We then performed an *in silico* genome analysis to assess the site specificity of sgRNA_del21 using Cas-OFFinder, followed by CCTop and COSMID. We could not detect off-target sites with either none or single mismatch and DNA bulge size=0. However, introducing 2 random mismatches led to the discovery of 2 potential off-targets, both located in intergenic sequences. When 3 random mismatches were introduced, we identified a total of 86 potential off-targets. The majority of these off-target sites were found in intergenic regions (41) and introns (44), with only one potential off-target located in a coding region (3' untranslated region of *ZNF641* gene). We focused on six off-targets out of these candidates, based on their potential relevance to gene function in the epidermis.

TIDE analysis outlined the presence of unwanted cleavage in one of the predicted off-target sites (Fig. S1B). These preliminary data suggested that the editing strategy designed to tackle the EBS01 mutation is indeed appropriate.

Lentiviral-mediated genome integration of the CRISPR/Cas9 components and their constitutive expression may increase off-target cleavage of similar genomic sequences overtime and trigger unwanted immunological response due to the Cas9 bacterial origin.[19] In fact, stable genome

integration of the lentiviral-mediated gene editing cassette is neither needed, nor desirable for clinical application, in that transient expression of both endonuclease and guide RNA is sufficient to attain a stable gene editing.

However, human keratinocytes are hard to transfect and genome access turns out to be a major issue. Thus, different transfection methods were attempted to deliver the gene editing machinery into EBS01 keratinocytes.

Plasmids expressing CRISPR/Cas9 components were initially delivered into cells using commercial lipofectamine reagents, which turned out to be highly inefficient. To implement the transfection efficacy and assure an optimal editing efficiency, additional electroporation procedures were investigated, all of which were highly toxic for normal human keratinocytes (data not shown).

To overcome these hurdles, EBS01 primary keratinocytes were directly electroporated with a ribonucleoprotein complex (RNP) composed of the SpCas9 endonuclease protein and the guide RNA specific for the mutant allele (sgRNA_del21) (sequence in Table S1).

Following RNP nucleofection, edited EBS01 keratinocytes (eEBS01) underwent further analysis to characterize genotypically and phenotypically the editing impact, always comparing them with a not transfected EBS01 sample.

Genomic DNA was extracted from sub-confluent eEBS01 and EBS01 cultures and the locus around the 21 base pair deletion was amplified by PCR and used to perform Sanger sequencing. The amplicon sequences were analysed using TIDE analysis (sensitivity >1-5%) and validated with next-generation sequencing (NGS).

As shown in Figure 2B, TIDE analysis outlined a strikingly high allele specificity of our tailored gene editing approach. In eEBS01 cells, the wild-type allele was virtually untouched, whereas the mutant allele displayed a significant amount of InDels abolishing the open reading frame and expression of

pathogenic K14. NGS analysis of three independent RNP nucleofections confirmed the remarkable allele specificity (Table S2), with a mutant allele specific gene editing greater than 95% (Fig. 2C).

Of note, ddPCR (Fig. S2A) and western blot analysis (Fig. S2B) showed that editing of the mutant allele restored both *KRT14* mRNA and K14 expression in eEBS01 keratinocytes, as compared to EBS01 cells. Gene editing specificity was confirmed in an agarose gel analysis, in which PCR products were amplified with oligonucleotides specific for the wild-type (268 bp) and mutant alleles (266bp). As shown in Figure 2D, the introduction of several mismatches, after editing and error prone DNA repair, caused the inability of the forward mutant allele specific oligonucleotide (KRT14_seq_del21 primer, see Table S1) to anneal in the edited region, determining the formation of a feebler eEBS01 mutant allele amplification product. Finally, NGS data were employed to calculate the frequency of deletion, insertion and substitution in eEBS01 keratinocytes, outlining deletion as the most frequent modification type (75%), whereas insertion and substitution were detected at a lower frequency (19% and 6% respectively) (Fig. 2E). An independent analysis of the NGS data was performed to gain insights into the prevalence of out-of-frame sequences (80% of the total sequences). The most frequent specific rearrangements are illustrated in Figure 2F.

Off-Target analysis supports RNP complex-mediated editing specificity and safety

To properly address safety issues, a comprehensive analysis assessing potential off-target sites was performed. Genomic DNA purified from sub-confluent EBS01 keratinocytes electroporated with the RNP complex was used to amplify and sequence off-target genomic regions previously identified after lentiviral transduction. TIDE analysis of eEBS01 shows no undesirable changes in any of the 6 predicted off-target sites, including *PPFIBP1*, thus recovering the unwanted cut chance determined by the genomic lentiviral vector-mediated integration and stable expression of the CRISPR/Cas9 components (Fig. S3).

Unbiased genome wide GUIDE-seq analysis was carried-out, the DNA library was sequenced using Illumina Miseq. The subsequent datasets were analysed using GUIDE-seq Bioconductor package software. The analysis confirmed preservation of the *KRT14* wild-type allele but outlined 9 "putative" off-target sites with a frequency below 3% but above 1% (Table S3). However, the vast majority of the implicated gene regions are either intergenic or intronic. The unique coding region involved is attributed to the *ZNF320* gene, whose expression is anyhow low in basal and superbasal keratinocytes and with a very low GUIDE-seq predicted editing rate. We NGS validated two off-target sites identified in intronic regions (*MIPOL* and *BAIAP*) and in the only coding region (*ZNF320*) detected with GUIDE-seq, for which we obtained appropriate quality PCR products. NGS showed editing efficiencies of 4.1%, 5.3%, and 6.9% for these respective targets (Table S3; NGS raw data published on GSE246345).

Overall, this data indicates the presence of a small number of potential off-target sites, which have a minimal effect on gene function, underscoring the safety of this non-viral CRISPR/Cas9 approach.

Editing of *KRT14* mutant allele restores functional intermediate filament network in EBS01 keratinocytes

Given the role of K5/K14 pairs in the assembly of keratinocyte intermediate filaments, we investigated whether the unmodified wild-type *KRT14* allele would suffice in restoring normal structural and functional phenotype in edited keratinocytes.

Healthy donor (NHEK), eEBS01 and EBS01 keratinocytes were seeded onto glass coverslips. Immunostained colonies clearly showed that corrected eEBS01 keratinocytes contained a properly functioning intermediate filament network, virtually indistinguishable from that of healthy NHEK. Keratin filaments were well organized and capable of branching throughout the plasma membrane. In contrast, EBS01 cells displayed a pathologic, roughly fragmentated keratin pattern (Fig. 3A). We have analysed the intermediate filament assembly in 636 NHEK, 722 EBS01 and 1090 eEBS01 cells.

Such analysis is summarized in Fig. S4 (panel D). The magnified areas shown in Fig. S4 (A-C) are strictly representative of the several images that we have analysed in several independent experiments. This data confirmed the efficacy of this gene editing approach in restoring EBS altered cellular phenotype Of note, the ablation of the mutant *KRT14* allele results in *KRT14* haploinsufficiency, which is comparable to the condition characterizing heathy carriers of recessive EBS mutations, who are completely asymptomatic.[20–22]

Additional functional assays evaluated the regain of mechanical strength in eEBS01 keratinocytes, which is mandatory for a more comprehensive validation of an appropriate functional correction.[23]

Heat shock assay is one of the easiest and most reproducible tests demonstrating the instability and thermal sensitivity of mutant keratins in EBS cells. The transient increase in thermal energy of the system results in evident depolymerization and impairment in affected keratinocytes' filament network remodelling, which may render cells vulnerable to cytolysis *in vivo* and support the increased EBS blisters formation in warm environments.[24]

To this end, EBS01, eEBS01 and NHEK keratinocyte colonies were submitted to thermal shock. At time zero and fifteen minutes after heat shock, EBS01 keratinocyte's intermediate filaments showed an increased disruption of keratin filaments, particularly nearby the nuclear region, which was partially recovered approximately 60 minutes after thermal shock, with aggregates limited to a small portion of the cytoplasm.[25,26] Such cytoplasmatic aggregates persistence was not observed in NHEK or eEBS01 (Figure 3B).

Since intermediate filaments also play a role in controlling cell mechanical stress, cultured epidermal sheets prepared from NHEK, EBS01 and eEBS01 were detached from the vessel by incubation with Dispase II protease (Fig. 3C). EBS01 cultured sheets disintegrated into small pieces when subjected to high inversion force, whereas eEBS01 sheet showed a structural compactness comparable to that of the healthy donor (NHEK) (Fig. 3D). The greater eEBS01 mechanical strength was also quantitatively evaluated counting sheet-derived fragments and confirmed that specific deletion of the

mutated *KRT14* allele confers to eEBS01 cells the capability to reassemble proper and resistant cohesive structures (Fig. 3E).

Edited eEBS01 cells revert the disease phenotype in skin equivalents

We have generated human skin equivalents containing dermal and epidermal compartments resembling morphological characteristics of human skin. This was achieved by cultivating keratinocytes on a decellularized human dermal matrix.

Figure 4A shows haematoxylin/eosin staining of sections of decellularized dermal matrixes without cells (Dermis) and those overlaid by a fully stratified epidermis (NHEK). Figure 4B (left panels) illustrates the decellularized dermal matrix seeded with EBS01 cells. The regenerated epidermis shows the presence of blisters within the epidermal basal layer (at arrows). In contrast, no blisters were observed in the epidermis generated by gene-edited eEBS01keratinocytes (Figure 4B, right panels).

In summary, these results show that the allelic-specific gene editing of mutant *KRT14* restores proper expression of K14 and functional intermediate filaments in primary clonogenic EBS keratinocytes.

CRISPR/Cas9-mediated gene editing via RNP complex electroporation preserves epidermal stem cells

Epidermal regeneration and repair processes rely on long-lived stem cells producing short-lived transient amplifying (TA) progenitors that eventually give rise to terminally differentiated keratinocytes. Keratinocyte stem cells and TA progenitors are located in the basal layer of all stratified epithelia and generate different clonal types, referred to as Holoclones and Meroclones/Paraclones respectively.[27–31] In view of future clinical applications, the essential feature of any cultured epithelial graft is an adequate number of Holoclones-forming cells, which are mandatory for a stable long-term regeneration of all squamous epithelia.[10,29,32–34] Clonal analysis of EBS01 clonogenic keratinocytes

confirmed the presence of each clonal type in the culture (Holoclones, Meroclones and Paraclones), excluding an impact of this *de novo* mutation on the EBS01 derived keratinocyte stem cell compartment.

To first investigate a potential impact of gene editing on the distribution of the different keratinocyte clonal types, we took advantage of single keratinocyte RNA sequencing analysis. To this end, we performed transcriptomic profile analysis of EBS01 and eEBS01 keratinocytes following the same pipeline recently published[28], obtaining 5,350 and 7,200 cells, respectively. As reported, both samples contained the previously identified 5 keratinocytes clusters (Fig. 5A-C), three of which expressed clonogenic markers (Holoclones, Meroclones and Paraclones clusters), the other two expressing differentiation markers (Terminally differentiated 1 and 2 clusters) (Fig. S5A-B). In particular, the Holoclone (stem cell) cluster displayed a "holoclone signature" able to distinguish it from the other clusters (Fig. S5A-B).

To further investigated whether Holoclones were preserved and properly corrected after the gene editing procedure, two clonal analyses of EBS01 and eEBS01 keratinocytes were performed, as described in Material and Methods. The classification of the clonal type confirmed the comparable percentage of Holoclones, Meroclones and Paraclones in both samples (Fig. 5D-E). Genomic DNA, analysed by PCR amplification using primers specific to amplified wild-type and mutant alleles (see Table S1) confirmed that holoclone-forming cells have been edited (Fig. 5F).

As shown in Supplemental Fig. S6, both clonogenicity (A) and percentage of aborted colonies (B) (and growth rate) were comparable in long-term cultures generated by EBS01 and eEBS01 keratinocytes, indicating the absence of clonal selection and/or selective advantages of eEBS01 keratinocytes over the EBS01 cells.

These data demonstrate that the gene editing procedure was able to preserve and edit the population of epidermal stem cells crucial to a future *ex vivo* gene therapy aimed at full epidermal restoration.

Discussion

Epidermolysis Bullosa simplex (EBS) is a rare mechanobollous disease inherited mainly in an autosomal dominant fashion and affecting a few thousands of people worldwide. Mutations affecting *KRT14* account for approximately 30% of the reported cases. Mutant *KRT14* exerts a dominant negative effect on the normal allele, perturbing the basal keratinocyte intermediate filament network and leading to intraepidermal blister formation. Hence, a great portion of EBS patients carrying *KRT14* mutations could potentially benefit from the correction of the genetic defect.[35]

Gene replacement strategies, whereby a functional copy of the defective gene is introduced in the genome of clonogenic keratinocytes, has been successfully exploited in other forms of EB, such as the *LAMB3*-dependent JEB and RDEB, which are recessively inherited.[6,8,10–13] However, gene replacement is not appropriate for the treatment of dominantly inherited genetic diseases, which instead require either the selective disruption of the mutated allele or the precise editing of the specific mutation.[36,37] An appropriately designed gene editing machinery would allow to discriminate and inactivate, via error prone NHEJ pathway, only the mutant allele, leaving the wild-type counterpart functionally intact. Gene editing has been employed to permanently to repair both dominant[38–41] and recessive[42–44] mutations related to EB using knockout and homologous recombination techniques. The end-joining pathways, often harnessed for gene knockout, represent the most efficient repair mechanisms for double-strand breaks.[45] Consequently, gene knockout is the most effective form of gene editing.

This work presents the first evidence of therapeutically relevant allele-specific genetic correction in primary cultures of EBS-derived epidermal stem cells. CRISPR/Cas9 based allele-specific editing platform using a guide RNA specific successfully edited a *de novo* c.475/495del21 mutation within exon 1 of *KRT14* gene in a dominant form of EBS. Our tailored approach was highly effective in disrupting the mutant *KRT14* allele on EBS01 primary keratinocytes (editing efficiency greater than 95%) with no editing on the wild-type allele. To ensure transient expression of editing machinery

suitable for clinical purposes, the sgRNA – Cas9 ribonucleoprotein complex (RNP) has been directly delivered to EBS01 keratinocytes. Since InDels generation at off-target sites sill poses a risk to the use of engineered nucleases, we also demonstrated a low occurrence of non-specific CRISPR/Cas9 mediated cleavages through unbiased GUIDE-seq analysis.

Thus, this approach succeeded in abolishing the mutant *KRT14* allele expression and the pathogenic keratin almost entirely, and fully restored a functional intermediate filament network. More importantly, we provide evidence that the long-lived self-renewing stem cells have been targeted and corrected by the gene editing machinery without any cytotoxicity, thus maintaining the ability to regenerate a virtually indistinguishable functional epidermis.

The selection of a gene editing tool for allele-specific genetic correction depends on factors such as the specific genetic mutation, the delivery method, the desired level of precision and, perhaps more importantly, on the cell type, mainly when specific somatic stem cells need to be targeted. In the case of EBS01 cells, the deletion of 21 base pairs (c.475/495del21) within exon 1 of *KRT14* led us to design a sgRNA tailored to specifically target the mutant allele. We opted for a gene editing approach utilizing SpCas9, known for its high cutting efficiency. We have introduced the sgRNA and SpCas9 as a ribonucleoprotein (RNP) complex that overcomes many of the challenges associate with mRNA delivery, as the translation steps and the folding of the Cas protein. The RNP complex is immediately active as it is fully developed. Emerging gene editing tools may offer more precise approaches than traditional methods, but often have an efficiency not sufficient to fully tackle a specific population of epidermal stem cells, which represent a small percentage of clonogenic keratinocytes. Promising gene editing tools for allele-specific genetic correction include prime editing[46,47], base editing[48,49], and Cpf1/Cas12-based editing[50–52] which provides versatility by targeting distinct DNA sequences, widening the scope of targetable genetic mutations. These tools hold great therapeutic promise due to their precision and reduced off-target effects.

In the context of clinical translation and safety of gene editing strategies, a key aspect is the analysis of off-target effects. As sequencing technologies and data analysis tools continue to advance, there may be, in the near future, more cost-effective and high-throughput options for off-target analysis than whole-genome sequencing.

Our study provides a clear demonstration of the efficacy and potential safety of an allele-specific CRISPR based gene editing approach, which we envision to further translate into a long-lasting decisive clinical treatment for patients suffering from EBS and possibly other related skin-blistering diseases. Although we did not observe abnormal clonal expansion, clinical translation would require additional efforts to determine the optimal RNP dosage to achieve maximal on-target efficiency and minimal off-target impact and thoroughly verify the absence of genotoxicity and genomic instabilities.

Materials and Methods

Patient, Clinal data and treatments

EBS patient (EBS01) displayed skin lesions to exclusively the acral regions and no mucosal blisters nor erosions were ever reported either by him or his parents. Clinical description and genetic counselling with genetic analysis of the family in Supplemental Materials and Methods.

Structural Modeling

The structure predictions were performed in a standalone platform of AlphaFold2 and AlphaFold-Multimer [15] as implemented in ColabFold, which was set up on a local computer with a Linux operating system and accelerated with two NVIDIA GeForce RTX 2080 Ti GPU. The “Template mode” using PDB 3TNU [16] was used for this purpose. The other parameters were kept at their default values**.**

Primary human cell culture from Epidermolysis Bullosa Simplex patient

A skin biopsy has been collected from the EBS01 patient, after obtaining the informed consent. Briefly, skin biopsy was minced and treated with 0,05% trypsin/ 0,01% EDTA for 4 h at 37°C. Every 30 min keratinocytes were collected, plated (2,5-3 x 10^4 cells/cm^2) on lethally irradiated 3T3-J2-Y cells and grown at 37°C, 5% CO2 in humidified atmosphere in KGM medium: Dulbecco's modified Eagle's (DMEM) and Ham's F12 media (2:1 mixture) containing fetal bovine serum (FBS) (10%), penicillin-streptomycin (50 IU/ml), glutamine (4mM), adenine (0,18mM), insulin (5mg/ml), cholera toxin (0,1nM), hydrocortisone (1,1mM), triiodothyronine (Lithyronine Sodium. 2nM), epidermal growth factor (EGF, 10ng/ml). When sub-confluent, cell cultures were serially propagated.

3T3-J2 cell line

Mouse 3T3-J2 cells were a gift of Professor Howard Green, Harvard Medical School (Boston MA, USA).[53] Fibroblasts were cultivated in DMEM supplemented with 10% g-irradiated donor adult bovine serum, penicillin-streptomycin (50IU/ml) and glutamine (4mM). GmbH, (Idar-Oberstein, Germany) produce a GMP clinical grade 3T3-J2 cell bank. That have been authorized for clinical use by national and European regulatory authorities.

Ribonucleoprotein (RNP) complex formation and Nucleofection

The synthetic guide RNA was designed straddling both the terminal sides of the c475/495del21 mutation (5'-GCTGAGGTTCAAGACCATTG-3') and directly flanking an "AGG" PAM. It was modified to drive the maximum editing efficiency (Invitrogen, #A35514, Table S1) and was mixed in a 1,1:1 molar ratio with the Cas9 protein (Alt-R S.p. Cas9 Nuclease V3, IDT, #1081058). 5 x 10^5 keratinocytes were resuspended in 100µl primary cell nucleofection solution (P3 Primary Cell 4D-Nucleofector Kit, Lonza, #LOV4XP3024), mixed with the RNP complex solution and 4µM Cas9 electroporation enhancer (Alt-R Cas9 Electroporation Enhancer, IDT, #1075915). Cells were

electroporated using a 4D-Nucleofector (4D-Nucleofector Core Unit, Lonza, #AAF-1001B; 4D-Nucleofector X Unit, Lonza, #AAF-1001X) using the program DS-138.

Editing analysis by sequence decomposition (TIDE)

eEBS01 and EBS01 keratinocytes genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, #51304). A 500 base pair region around eEBS01 and EBS01 genomic target site was amplified by PCR (primers in Table S1). PCR amplicons were subjected to conventional Sanger sequencing. The resulting sequence trace files were uploaded on TIDE web tool with the guide RNA sequence as input.

PCR and allele characterization

Screening of the allele pattern, in eEBS01 and EBS01 keratinocyte, was done by PCR. The "KRT14_seq_del21" forward primer was specific only for the mutant allele in EBS01 keratinocyte, "KRT14_seq_wt" forward primer was specific only for the wild-type allele. Reverse primer was specific for a sequence common to both alleles (Table S1).

OFF-target analysis

CRISPR/Cas9 online predictors were used to identify the genomic regions which may present the greatest probability of off-target cuttings. Off-target probability was evaluated on the basis of mismatches numbers and the genomic loci of the most probable in silico off-target sites were sequenced and analysed. The resulting sequence trace files were uploaded on TIDE web tool with the guide RNA sequence as input.

NGS analysis

The region near the target site was amplified by specific PCR primers with sequence adaptor (Supplementary table 1, KRT14ex1_NGS and KRT14intr1_NGS) and 25 µl of purified amplicon was used to NGS analysis using Illumina sequencing platform. The clipping of reads was performed using

Trimmomatic (v 0.36) and paired-end reads were merged using software FLASH2 (v 2.2.00) to obtain a single, longer read that covers the full target region. The processed reads were mapped, using BMWA MEM (v 0.7.15), to the reference sequence (the wild type *KRT14* exon1 sequence) with default alignment parameters. Only high quality, merged, on target reads were considered for further processing. Finally, the identification and quantification of sequences alleles using CRISPResso (v 1.0.13) occurred. The NGS raw data are available in Gene Expression Omnibus with accession number GSE246345.

Clonal analysis

Sub-confluent keratinocytes mass cultures were trypsinized and 0,5-1 cell was plated into each well of a 96-well plate after serial dilution. After 7 days of cultivation, single clones were identified under an inverted microscope and treated with 0,05% trypsin and 0,01% EDTA at 37° for 15-20 minutes. One quarter of the clone was plated onto a 100mm indicator dish, cultivated for 12 days and stained with Rhodamine B for the classification of clonal type. The remaining three quarter was sub-cultivated on an adequate plastic support and used for further analyses.[34]

Immunofluorescence

Keratinocytes were plated at 2500 cell/well onto glass coverslips. After the formation of small colonies, cells were fixed with ice cold Methanol-Acetone (1:1) at -20°C for 10 minutes. Cells were permeabilized with PBS/Triton 0,5% for 15 minutes. Blocking solution (BSA 5%, 0.3% Triton in 1X PBS) was added for 30 minutes at 37°C and sections were incubated with primary and secondary antibody (Table S1). Cell nuclei were stained with DAPI. Fluorescence signals were monitored under a Zeiss AXIO Imager A.1 Manual Operation Fluorescence Microscope with EC Plan-Neofluar 40X/0,75 objective.

GUIDE-seq analysis

5 x 10^5 primary EBS01 keratinocytes were nucleofected (as described in RNP paragraph) with the RNP complex and 40nM of the annealed dsODN. Treated keratinocytes were then plated (4-6 x 10^3 cells/cm2) on lethally irradiated 3T3-J2 cells and cultured until sub-confluence. Keratinocytes' genomic DNA was extracted and 14µg was sent to Creative Biogene for GUIDE-seq library preparation, sequencing in order to identify RGN (CRISPR RNA-guided nucleases)-dependent and -independent genomic breakpoint "hotspots". DNA library was sequenced using Illumina Miseq. The subsequent datasets were analysed using the *GUIDEseq* Bioconductor package software.

Encapsulation with 10X Genomics chromium system and bioinformatic analysis on single-cell RNA-seq data

Fully confluent keratinocytes were detached and cells were accurately resuspended to obtain a single cell suspension. About 10.000 cells of each eEBS01 and EBS01 samples were loaded into two channels of the Chromium Chip B using the Single Cell reagent kit v3.1 (10X Genomic) for Gel bead Emulsion generation. Following capture and lysis, cDNA was synthesized and amplified. Fifty nanograms of the amplified cDNA were then used for each sample to construct Illumina sequencing libraries. Sequencing was performed on the NextSeq550 Illumina sequencing platform following the 10X Genomics instruction for read generation, reaching at least 50000 reads as mean reads per cell.

For the bioinformatic analysis, the Cell Ranger pipeline (version 3.1.0) was used to generate FASTQ files, to align reads to the reference transcriptome (GRCh38) and to calculate UMI counts from the mapped reads. Expression data were imported in R version 3.6.3 and analyzed using Seurat[54] (version 3.1.5) R package. Cells were classified using an annotated scRNA-seq dataset of human keratinocytes[28] as reference and the FindTransferAnchors and TransferData functions in Seurat with default parameters. We assessed the quality of the assigned labels monitoring the expression of known markers. Expression data are available in Gene Expression Omnibus with accession number GSE246345.

Heat shock assay

Keratinocytes were plated at 10000 cell/well onto glass coverslip. Cells culture medium was replaced with KGM medium at 43°C and the well plate was immediately placed in a water bath set at 47°C. After 15 minutes of heat stress, the medium was immediately replaced with fresh KGM medium at 37°C and the cells were allowed to recover in the incubator at 37°C, 5% CO2 in humidified atmosphere. Coverslips were removed at 15-minute intervals thereafter and immunostained.

Dispase-based keratinocyte dissociation assay (DDA)

4-6 x 10^3 cells/cm^2 keratinocytes were plated, on lethally irradiated 3T3-J2 cells onto a 6 well plate and cultured. After 20 days, the epidermal sheets were washed with grafting wash and incubated with Dispase II (Roche, #0494207801), 2,5U/ml diluted in PBS, for 1 hour at 37°C. After detachment, one sheet was subjected to low force stress with orbital rotation (200 rpm) for 5 minutes at 37°C and the other monolayer was transferred in a 15ml Falcon tube with 5ml of 1X PBS and exposed to high mechanical stress by 20-50 inversions. Fragments count was performed with ImageJ.

Decellularized dermal matrixes preparation, cryopreservation and sectioning.

Decellularized human dermal matrixes were obtained using human skin samples from surgical waste (abdominoplasty or mammoplasty). Briefly, skin biopsies were sectioned in fragments of approximately 1,5 cm^2, immersed in sterile PBS at 60°C for thirty seconds under constant stirring and then in sterile cold PBS for 1 minute. The epidermis was then mechanically detached from the dermis using forceps. After decellularization, samples were rinsed in KGM for 24 hours. The following day, the decellularized dermal matrixes were seeded with primary human keratinocytes (1 x 10^5 cells per scaffold) onto lethally irradiated 3T3-J2 cells (5 x 10^4 cells per scaffold) in KGM. After 10 days in submerged culture, the media was carefully removed, and the samples were gently moved in millicell® cell culture (Merk) and were further cultured for 20-24 days in air-liquid interface (ALI) condition to induce epidermal differentiation. 3D human skin equivalents were dehydrated in

a sucrose gradient 0.9M and 2M for thirty minutes respectively at RT, embedded in Killik-OCT cryostat embedding medium (Bio-Optica) and frozen. 7µm sections of embedded skin equivalents were obtained with a histological cryomicrotome (Leica CM1850 UV).

Haematoxylin and eosin staining.

Hematoxylin and eosin staining was performed on 7µm cryosections of decellularized dermal matrixes (Harris haematoxylin for 1 min, running tap water for 1 min, eosin Y 50% in ethanol for thirty seconds, 95% ethanol for 1 min, 100% ethanol for 1 min, two rinses in fresh 100% ethanol for 1 min each) and observed with Zeiss Microscope Axio Imager M2 with an EC Plan-Neofluar 10X/0.3 M27 air objective.

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Author Contributions

C.C. performed experiments, analyzed data, assembled all input data. E.E. defined single cells RNA seq analyses and revised the manuscript. L.D.R. defined and analyzed functional assay and revised the manuscript. L.S. performed 3D skin equivalent assays. F.C. performed clonal analysis. M.F. and S.B. conducted bioinformatics analyses. A.P. performed protein structure prediction and modeling. G.B. made scRNA-seq library. E.T. performed NGS to identify gene variants. A. Paganelli, C.F. and C.M. provided EBS01 patient clinical management. M.C.L. and M.D.L. coordinated the study, defined strategic procedures, administered the experiments, and wrote the manuscript.

Conflict of Interest

M.D.L. is co-founder and member of the Board of Directors of Holostem Terapie Avanzate (HTA) s.r.l in liquidation, Modena, Italy, as well as consultants for J-TEC-Japan Tissue Engineering, Ltd. The other authors state no conflict of interest.

Keywords

Epidermolysis bullosa, genetic disease, gene therapy, stem cells, keratinocytes biology, gene editing

Data Availability Statement

Data availability Sequencing data have been deposited to Gene Expression Omnibus with accession number GSE246345. We declare that the data supporting the findings of this study are available within the paper and its Supplementary Information Files or from the authors upon request.

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656

657 **Figures legends**

658 **Figure 1**: **A novel monoallelic *KRT14* deletion causing a dominant form of Epidermolysis**
659 **bullosa simplex**

660 **A)** Clinical images of the patient EBS01: bullous lesions are present on the palmoplantar region.
661 Unremitting blister on the heel, dorsal medial part of the left foot and right peri-malleolar region are
662 shown in the panel below. **B)** Amino acid sequence of the wild-type and mutant K14. The invariant
663 residues are displayed in black boxes and the red box highlights the mutant region with the 7 amino
664 acids deletion. The secondary structures are represented above the sequences as ribbons (helices)
665 and arrows (strands), respectively; blanks indicate unassigned regions. The Head and Tail domains
666 of *KRT14* are represented as N-terminal and C-terminal grey boxes, respectively. Coil and Linker
667 regions are represented as yellow and cyan boxes, respectively. **C)** Predicted three-dimensional
668 structures of human wild-type (yellow) and mutant (grey) K14 protein. The lack of the Linker 1
669 region, which is visible in the c475/495del21 protein, is circled in red.

670 **Figure 2: Gene-editing of *KRT14* mutated allele**

671 **A)** Representation of the EBS01 patient's genotype and allele profile of *KRT14* gene on human
672 chromosome 17. The expanded section of the exon 1 illustrates the 21 nucleotides absent in *KRT14*
673 exon 1 mutant allele (light blue). Mutant allele specific guide RNA is depicted in purple, straddling
674 both the terminal sides of the deletion, and the -AGG- PAM sequence in red. **B)** Graphic
675 representation of TIDE analysis. Both the wild-type and mutant allele were detected in the untreated
676 sample (EBS01). In the treated sample (eEBS01) the mutant allele has been deleted by gene editing.

677 **D)** Histogram representation of NGS analysis of three technical replicates post gene editing. Edited
678 and not edited percentages on both wild-type and mutant alleles are calculated on the total number
679 of reads. **D)** PCR analysis of three technical replicates (EXP1, EXP2, EXP3). For each experiment,
680 a PCR product was amplified both from the EBS01 and eEBS01 keratinocytes' genome with primers
681 specific to anneal to wild-type (268 bp) and mutant alleles (266bp). **E)** Graphic representation of
682 editing derived InDels in mutant *KRT14* allele (N= 3). **F)** NGS analysis of PCR products
683 surrounding the Cas9 target sites in the genome of eEBS01 showed a wide variety of Indel mutations
684 mediated by NHEJ at the targeted exon 1. The top sequence is the mutant allele unmodified
685 sequence, the dotted line represents the Cas9 cleavage upstream of the PAM.

686 **Figure 3: Restoration of intermediate filaments**

687 **A)** Immunofluorescent staining of K14 intermediate filaments in healthy donor derived
688 keratinocytes (NHEK), untreated and treated EBS01 keratinocytes (EBS01 and eEBS01
689 respectively). Nuclei are stained in blue by DAPI. Scale bar= 20μm. **B)** Immunofluorescent staining
690 of K14 filament before and after heat shock assay. At time 0 and after 15 minutes recovery from
691 heat shock, NEHK and eEBS01 do not show any changes in the organization of cytoplasmatic
692 network, whereas EBS01 keratinocytes display disruption of intermediate filament also in the
693 perinuclear region. After 60 minutes recovery, EBS01 intermediate filament aggregates revert to a
694 condition similar to that before the hear shock. Nuclei are stained in blue by DAPI. Scale bar=
695 20μm. **C)** Dispase-mediated epidermal sheet dissociation assay of NHEK, EBS01 and eEBS01
696 epidermal cultures before (C) and after (D) mechanical stress. No fragmentation was detected in
697 samples subjected to low-force orbital rotation. In contrast, high force stress induced fragmentation
698 of EBS01, but not NHEK and eEBS01 sheets (**D**). **E)** Number of fragments derived from each
699 sample has been counted using ImageJ software and unpaired t-test showed a statistically significant
700 P-value between EBS01 and eEBS01 number of fragments (N=3; asterisk indicate P-value<0,05).

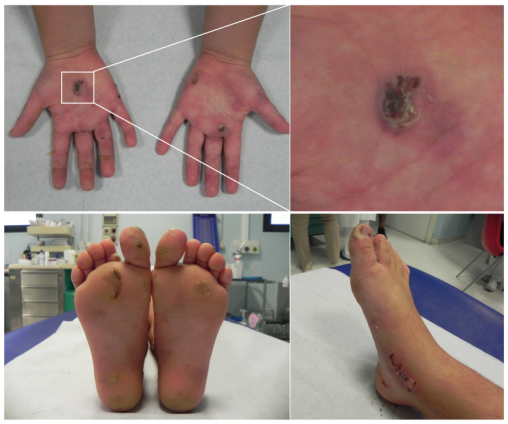
701 **Figure 4: 3D skin equivalents**

(A) Haematoxylin and eosin staining of sections (7-μm thick) of decellularized dermal matrixes (Dermis) seeded with normal human epidermal keratinocytes (NHEK). (B) The decellularized dermal matrix was seeded with EBS01 or eEBS01 cells. Black arrowheads show blisters originated in the epidermal basal layer only in EBS01 3D cultures. Scale bar, 100μm.

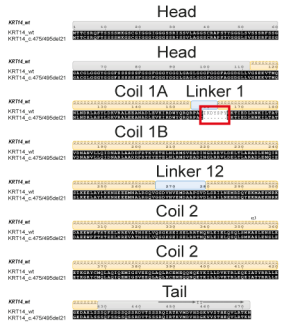
Figure 5: Genetic correction of epidermal stem cells

A-B) Uniform manifold approximation and projection (UMAP) of the scRNA-seq experiment. EBS01 and eEBS01 keratinocytes mass cultures were profiled, integrated and classified in all their clonogenic and differentiated clusters (H in red, M light blue, P grey, TD1 light brown, TD2 brown) Distribution and extension of the clusters are comparable between the two samples. Feeder layer derived fibroblast and low-quality keratinocytes are shown in light gray. **C)** Table shows the percentages of cells in each EBS01 and eEBS01 clusters after scRNA-seq analysis. This quantitative analysis demonstrates a comparable percentage between the treated and not treated samples. **D)** Graphical representation of clonal analysis assay results. Clonal population percentages represent the average value of Holoclones (H), Meroclones (M) and Paraclones (P) of two independent experiments (EBS01: Holoclones 5%, Meroclones 52%, Paraclones 43%; eEBS01: Holoclones 5%, Meroclones 48%, Paraclones 47%). As shown, there are no differences in clonal populations between EBS01 and eEBS01 keratinocytes. **E)** Representative indicator dishes for EBS01 and eEBS01 Holoclones, Meroclones and Paraclones. **F)** PCR products of holoclones eEBS01's wild-type and mutant alleles. Wild-type alleles result untouched, whereas all the mutated alleles display editing and lack of primer annealing and amplification. Not treated EBS01 sample was used as control (CP) in which both the wild-type and mutant allele are present and amplified.

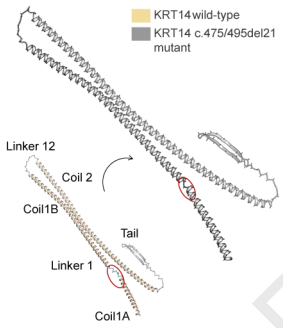
A

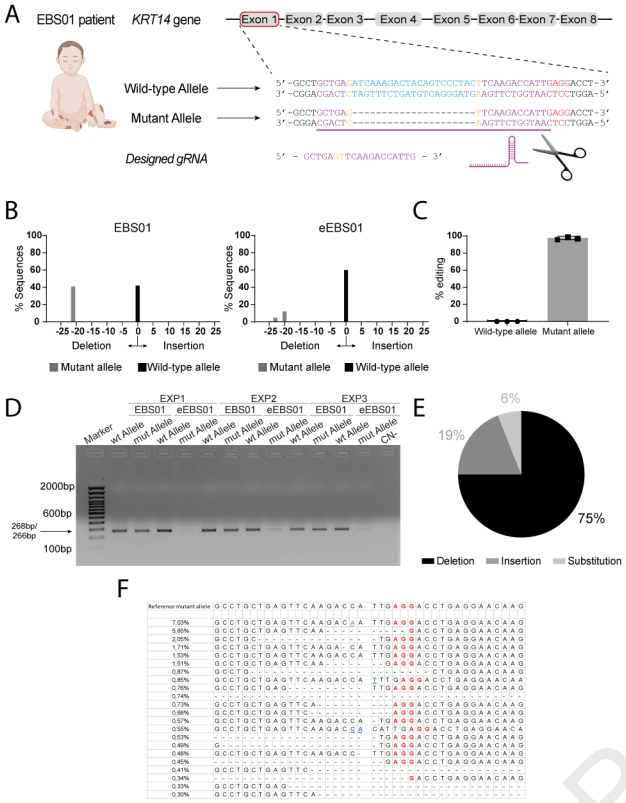


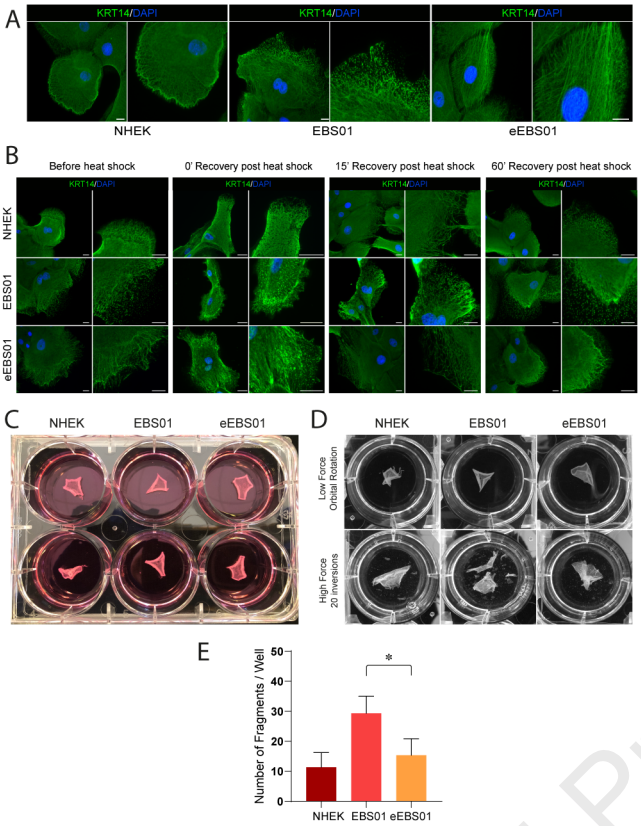
B Keratin 14 amino acid sequence

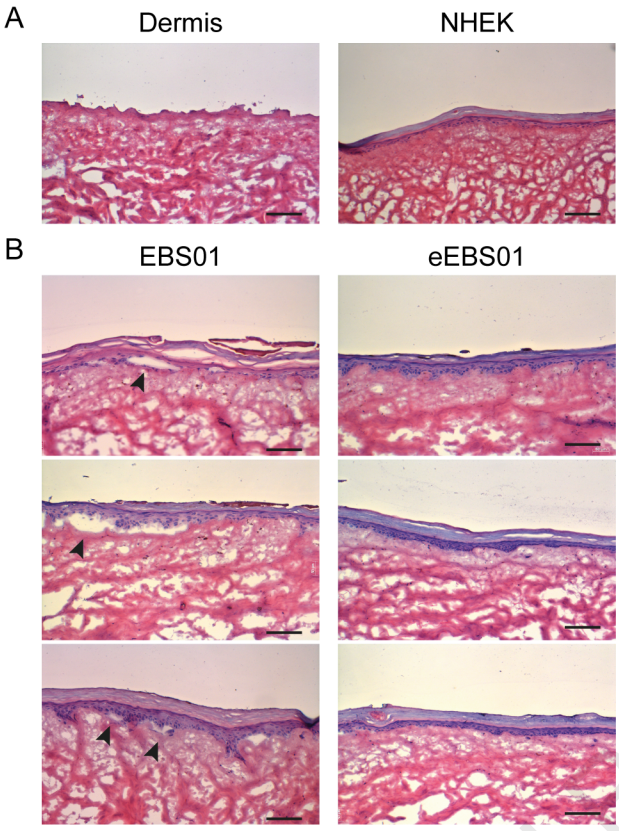


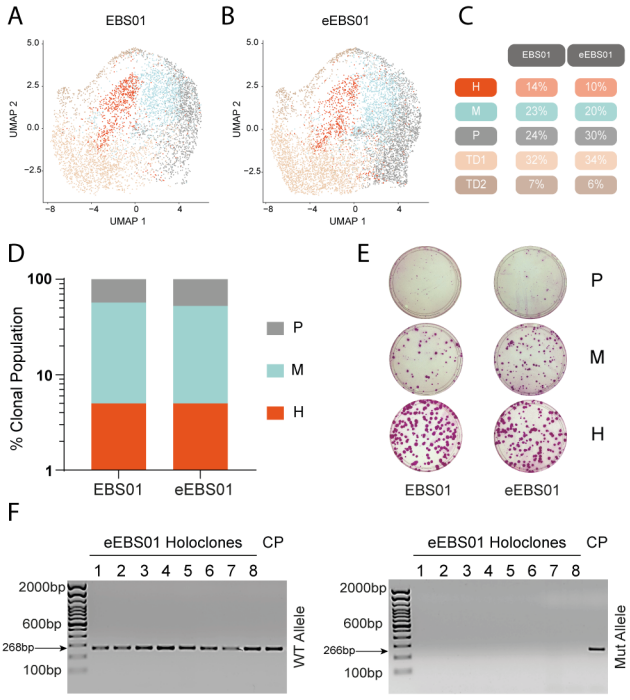
C Keratin 14 protein structure











De Luca and colleagues propose an allele specific gene editing strategy for EBS01 patient affected by a rare skin disease inherited in an autosomal manner (EBS). This work aims to restore a normal cellular phenotype and a correct intermediate filament network in epidermal stem cells.