CRISPR-Cas9–Based Genomic Engineering in Keratinocytes: From Technology to Application

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CRISPR-Cas9 is the most straightforward genome-editing tool to date. However, its implementation across disciplines is hampered by variable genome-editing efficiencies, reduced cell viability, and low success rates in obtaining clonal cell lines. This review aims to recognize all CRISPR-Cas9–related work within the experimental dermatology field to identify key factors for successful strategies in the different keratinocyte (KC) cell sources available. On the basis of these findings, we conclude that most groups use immortalized KCs for generating knockout KCs. Our critical considerations for future studies using CRISPR-Cas9, both for fundamental and clinical applications, may guide implementation strategies of CRISPR-Cas9 technologies in the (experimental) dermatology field.

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Introduction to CRISPR-Cas9 as a genomic editing tool

CRISPRs were known in the bacterial genome as hypervariable loci typically consisting of direct repeats, separated by sections of variable sequences called spacers, in the proximity of *CRISPR-Cas* genes. The mechanism of the CRISPR-Cas system to specifically target DNA for genome editing was utilized successfully for the first time in mammalian cells almost a decade ago (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013), and the functions as described extensively (Doudna and Charpentier, 2014) and schematically visualized in Figure 1a. Many bacterial species have variants of CRISPR and *Cas* loci, with the most extensively investigated variant as a genome-editing tool being the CRISPR-Cas9 system (Makarova et al., 2011).

CRISPR-Cas9–mediated genome editing requires a Cas9–guide RNA (gRNA) complex containing Cas9, CRISPR RNA (crRNA), and trans-activating CRISPR RNA (tracrRNA) (see Box 1: CRISPR Terminology). The complex can be introduced to target cells by various methods, as reviewed before (Lino et al., 2018; Shi et al., 2021). By the guidance of crRNA, the complex binds to complement DNA accompanied by a flanking protospacer adjacent motif 5′-NGG-3′ for *Streptococcus pyogenes* Cas9 (Chylinski et al., 2013). The Cas9–gRNA complex induces a double-stranded break at the target site (Deltcheva et al., 2011; Shah et al., 2013), which can be repaired by the target cell through either nonhomologous end joining (NHEJ) (Hefferin and Tomkinson, 2005) or homology-directed repair (HDR) (Liang et al., 1998). In NHEJ, the broken DNA strands are religated, either directly or after random nucleotide insertions or deletions (Takata et al., 1998). Often, this leads to frameshift mutations and premature stop codons, and therefore, this mechanism is readily used to knock out protein expression of interest. In HDR, the double-stranded breaks are repaired with the use of a sister chromatid as a homologous template strand. By multiple crossovers, DNA synthesis, and ligation, the damaged strand can be precisely repaired (Takata et al., 1998). Instead of a sister chromatid as template strand, an exogenous DNA template harboring the desired mutation or gene cassette can be introduced as single-strand or double-strand DNA, with homologous arms on the outsides (Chen et al., 2011; Radecke et al., 2010; Rouet et al., 1994).

Over the years, an increasing number of studies in the field of experimental dermatology harnessed the CRISPR-Cas9 toolbox, although current numbers are limited but increasing over the past 5 years (Figure 1b and c and Table 1). This review aims to recognize all the CRISPR-Cas9 work performed in human epidermal keratinocytes (KCs) to identify the best practices and key determinants for successful strategies in different human KC cell sources available, accompanied by critical considerations for future studies using CRISPR-Cas9, both for a fundamental and clinical application.

Delivery of the CRISPR-Cas9 machinery into KC

Cationic vectors, lentiviral vectors, or adenoviral vectors are mostly utilized for transducing the expression of Cas9 and a specific gRNA. Lentiviral vectors especially designed for this purpose, such as lentiCRISPR v2 deposited by Feng Zhang's laboratory (Sanjana et al., 2014), are readily available

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Abbreviations: 3D, three-dimensional; AAV, adeno-associated virus; COL7, type VII collagen; EB, epidermolysis bullosa; hPSC, Human pluripotent stem cell; HPV16, human papillomavirus type 16; iKC, induced keratinocyte; iPSC, induced pluripotent stem cell; IV, ichthyosis vulgaris; JEB, junctional epidermolysis bullosa; KC, keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa

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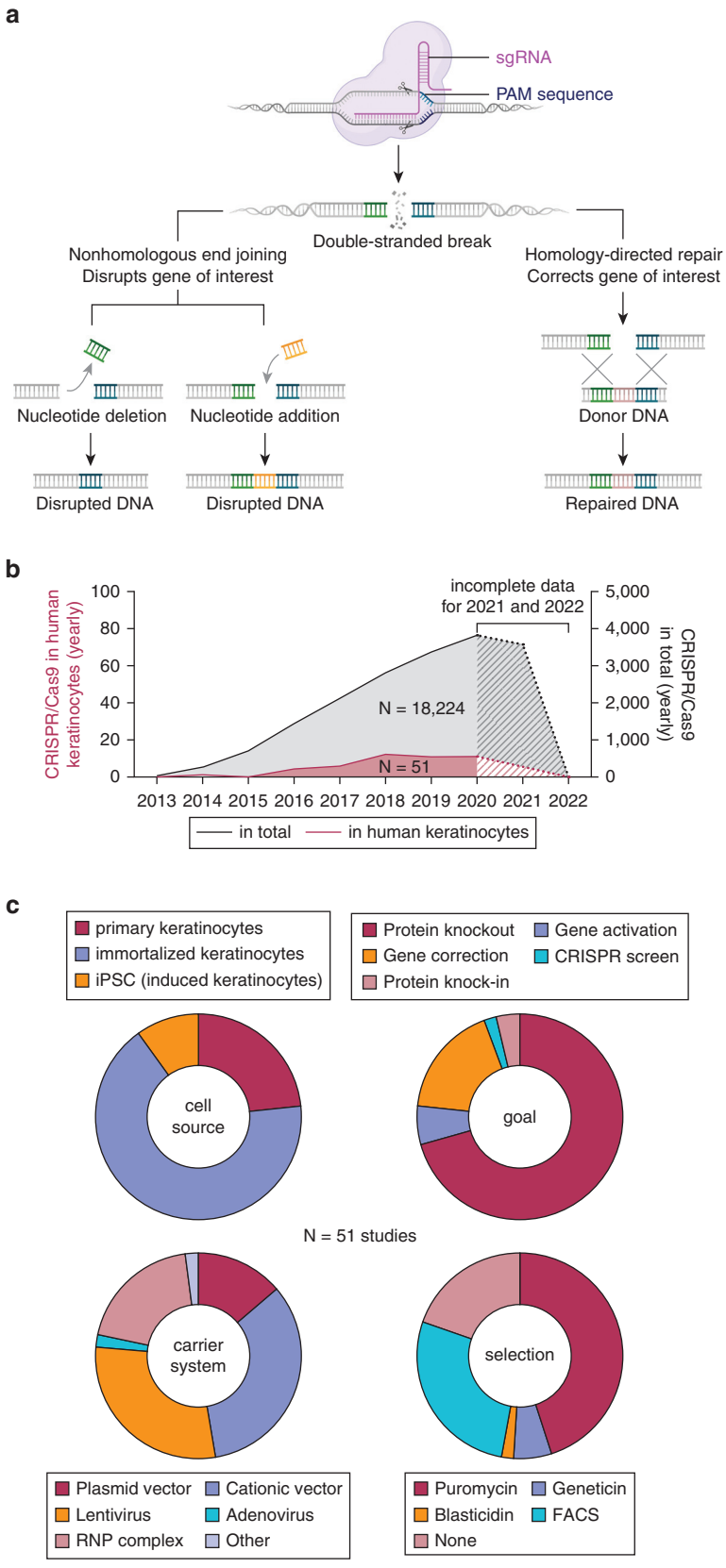

Figure 1. CRISPR-Cas9–initiated genomic repair in human keratinocytes. (**a**) Schematic overview of CRISPR-Cas9 mechanism (created with BioRender.com). (**b, c**) Graphical representation of publications using CRISPR-Cas9 in human keratinocytes, split by cell source, experimental goal, carrier system applied, and selection (marker) deployed. iPSC, induced pluripotent stem cell; PAM, protospacer adjacent motif; RNP, ribonucleoprotein; sgRNA, single-guide RNA.

Box 1. CRISPR Terminology

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas9	CRISPR-associated protein 9
Cas9n	Cas9 nickase
dCas9	Deactivated Cas9
PAM	Protospacer adjacent motif
crRNA	CRISPR RNA
tracrRNA	Trans-activating CRISPR RNA
(s)gRNA	(Single) guide RNA
RNP	Ribonucleoprotein
HDR	Homology-directed repair
NHEJ	Nonhomologous end joining

through Addgene (Watertown, MA) (plasmid #52961) and are easily amendable to encode the gRNA sequence(s) of interest. Lentiviral infection is often very efficient and leads to random incorporation of the encoded DNA into the infected cell's genome, causing a permanent transfer—and often also permanent induction—of Cas9 and the encoded gRNA sequence. Consequently, the constitutive expression of Cas9 and gRNA increases the risk of off-target cleavage of DNA, potentially leading to unforeseen genomic changes. In addition, lentiviral delivery can result in unwanted gene rearrangements and transgene silencing (Lino et al., 2018). The use of adenovirus over lentiviruses is preferred, as adenoviruses do not integrate easily into the genome (Stephen et al., 2010). Both lentivirus and adenovirus can induce strong immunogenic responses (Nayak and Herzog, 2010; Zaiss and Muruve, 2008), complicating their suitability for in vivo therapeutic use. Therefore, adeno-associated virus (AAV) particles, which show limited immunogenicity compared with adenovirus vectors (Zaiss et al., 2002), might be more suitable. Nevertheless, the drawback of AAV is that these particles have a smaller loading capacity than adenoviruses and lentiviruses, which can limit their use with relatively large plasmids encoding such as gRNAs and Cas9.

Electroporation or transfection of Cas9 and gRNAs, either as plasmids, mRNA, or ribonucleoprotein (RNP) complexes, is nowadays often used in immortalized KCs (Table 1). These delivery methods are easy to use and can be highly efficient (especially electroporation of RNP complexes), and the transient expression of gRNAs and Cas9 limits the risk for off-target effects.

CRISPR-Cas9 in human primary KCs

To study protein function, biological processes, or disease mechanisms, experimental cell or tissue culture models often include primary epidermal KCs of healthy individuals taken from excess skin that was removed during surgical procedures. Genetic predispositions are key in the pathogenesis of many skin diseases, from the obvious monogenetic to complex polygenic and multifactorial diseases. For example, ichthyosis vulgaris (IV) and epidermolysis bullosa (EB) are the results of homozygous (or compound heterozygous) mutations in *FLG* (for IV) and type VII collagen (COL7) gene *COL7A1* and *LAMB3* (both for EB) (Floeth and Bruckner-Tuderman, 1999; Ryynänen et al., 1991; Smith et al., 2006; Thyssen et al., 2013). Through genomic engineering, models for these monogenetic skin diseases can be created, allowing to study the contribution of the genetic risk factors in an in vitro setting against nonengineered KCs with an identical genetic background. Potential gene therapy strategies can be developed and validated for use in vitro and eventually in vivo. So far, CRISPR-Cas9 has been used in primary KCs, mainly to knockout or correct genes, as shown in Table 1.

In 2018, a protocol for the generation of knockout human primary KCs was published (Fenini et al., 2018a). To increase the lifetime of human primary cells, they were cocultured with 3T3-J2 fibroblasts as feeder cells in the presence of proliferation-enhancing ROCK inhibitor Y-27632 (Gandham et al., 2013), whereas the CRISPR-Cas9 machinery is delivered through lentiviral transduction of plasmid DNA, including a puromycin resistance cassette. Selection of modified KCs was performed on mitotically inactivated and puromycin-resistant fibroblasts. The modified KCs were still able to differentiate and were able to form three-dimensional (3D) skin equivalents (Fenini et al., 2018b; Grossi et al., 2020). In the studies mentioned earlier, antibiotic resistance was often conferred, allowing for the selection of KCs that were successfully infected. These KCs did not undergo successful genomic editing per se. In other words, the generation of isogenic clonal cell lines that harbor precisely the intended mutations is preferred to using selection procedures that will result in a mixed cell population with unspecified genomic alterations. Indeed, clonal expansion of primary KCs is a challenge given the limited lifespan. Nevertheless, EB-derived patient KCs, grown on feeder fibroblast cells and in the presence of Y-27632, were successfully targeted by CRISPR-Cas9 (Bonafont et al., 2021, 2019). Others circumvented the proliferative limitations by immortalizing the genetically altered primary KCs using a retroviral vector carrying human papillomavirus type 16 (HPV16) genes *E6* and *E7* before grafting experiments and organotypic 3D cultures for studies on junctional epidermolysis bullosa (JEB) (Benati et al., 2018) or Netherton's syndrome (Gálvez et al., 2020).

Most research utilizing CRISPR-Cas9 in primary KCs is focused on EB using patient-derived EB KCs, as reviewed recently (Kocher and Koller, 2021). In EB, the connection between the dermis and the epidermis is fragile, leading to severe clinical features such as blistering and subsequent debilitating infections. Using CRISPR-Cas9–induced HDR, the COL7 gene *COL7A1* in KCs derived from patients with recessive dystrophic EB (RDEB) (Bonafont et al., 2021; Hainzl et al., 2017; Izmiryan et al., 2018; Kocher et al., 2021) and fibroblasts derived from patients with RDEB (Kocher et al., 2021) can be restored, leading to re-expression of COL7. The COL7-corrected KCs were able to develop into high-quality skin equivalents when transplanted onto immunodeficient mice. Others showed that the use of dual single gRNA (sgRNA)-guided Cas9 nuclease can restore the *COL7A1* reading frame and reinstate the expression of COL7 in the KCs derived from patients with RDEB, enabling long-term regeneration of high-quality, properly adhesive skin after grafting onto immunodeficient mice (Bonafont et al., 2019). For JEB, a similar approach was successful: primary KCs carrying the homozygous *LAMB3* mutation in exon 14 were immortalized and corrected by HDR through an adenoviral vector carrying Cas9 and gRNA cassettes and a lentiviral

Table 1. Characteristics of Studies that Utilize CRISPR-Cas9 in Human Keratinocytes

Cell source	Publication	PMID	Cell Types (All Human)	Research Goal	Method of Introduction	Carrier	Cas9 Version	Repair	Selection
Primary keratinocytes	Nöske et al. (2016)	26828486	Adult primary keratinocytes	Protein knockout	Electroporation	Plasmid vector	SpCas9	NHEJ	FACS
	Yue et al., 2017	28777946	Foreskin primary keratinocytes	Gene activation	Electroporation	Plasmid vector	hCas9 D10A	HDR	Puromycin
	Hainzl et al., 2017	28800953	RDEB primary keratinocytes	Gene correction	Xfect	Cationic vector	SpCas9	HDR	Puromycin and blasticidin
	Kocher et al., 2017	28888469	Adult primary keratinocytes	Protein knockout	Electroporation	Plasmid vector	SpCas9	NHEJ	Blasticidin
	Fenini et al., 2018a	29287762	Adult primary keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
	Fenini et al., 2018b	30096351	Adult primary keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
	Izmiryan et al., 2018	30195791	RDEB primary keratinocytes	Gene correction	IDLV	Lentivirus	SpCas9	HDR	None
	Liu et al., 2018	30225000	Adult primary keratinocytes	Protein knockout	FuGene HD	Cationic vector	SpCas9	NHEJ	Puromycin
	Slivka et al., 2019	30938974	Adult primary keratinocytes	CRISPR screen	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin and blasticidin
	Herter et al., 2019	30594489	Adult primary keratinocytes	Gene activation	Lipofectamine 2000	Cationic vector	dCas9	n/a	FACS
	Jozic et al., 2019	31409528	Adult primary keratinocytes	Protein knockout	FuGene HD	Cationic vector	SpCas9	NHEJ	Puromycin
	Grossi et al., 2020	31502220	Adult primary keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
Immortalized keratinocytes	Liu et al., 2016	26228041	HPV16-transformed foreskin primary keratinocytes	Protein knockout	Lipofectamine 2000	Cationic vector	SpCas9	NHEJ	Puromycin
	Dahlhoff et al., 2017	28805349	HaCaT keratinocytes	Protein knockout	Lipofectamine 3000	Cationic vector	SpCas9	NHEJ	FACS
	Gao et al., 2017	28588028	HaCaT keratinocytes	Protein knockout	pLKO.1-puro	Lentivirus and adenovirus	SpCas9	NHEJ	Puromycin
	Sarkar et al., 2018	30021804	N/TERT foreskin keratinocytes	Protein knockout	TransfeX	Cationic vector	pSpCas9	NHEJ	Geneticin
	Swindell et al., 2018	29434599	N/TERT foreskin keratinocytes	Protein knockout	TransfeX	Cationic vector	pSpCas9	NHEJ	FACS
	Trothe et al., 2018	30252954	HaCaT keratinocytes and adult primary keratinocytes	Protein knockout	Ad5-CMV-Cas9 and Ad5-U6-sgRNA	Adenovirus	SpCas9	NHEJ	None
	Benati et al., 2018	30122422	Immortalized JEB adult primary keratinocytes	Protein knockout	IDLV	Lentivirus	SpCas9	NHEJ	None
	Chiang et al., 2018	29263274	HaCaT keratinocytes	Protein knockout	pSicoR-CRISPR-PuroR	Lentivirus	SpCas9	NHEJ	Puromycin
	Sawatsubashi et al., 2018	29330493	HaCaT keratinocytes	Protein knockin/ knockout	Electroporation	Plasmid vector	SpCas9	NHEJ	Puromycin
	Sun et al., 2018	29807809	HaCaT keratinocytes	Protein knockout	DNAJA4-gRNA-EGFP and Cas9-puro	Lentivirus	SpCas9	NHEJ	Puromycin
	Zhong et al., 2018	30132045	HaCaT keratinocytes	Protein knockin	GenJet	Cationic vector	SpCas9	HDR	Geneticin
	Baida et al., 2018	30410676	HaCaT keratinocytes	Protein knockout	RNAi-Max	RNP complex	SpCas9	NHEJ	None
	Bonafont et al., 2019	30930113	Immortalized adult primary keratinocytes	Protein knockout	Electroporation	RNP complex	SpCas9	NHEJ	None
	James et al., 2019	31391281	N/TERT foreskin keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
	Hatterschide et al., 2019	32581101	Foreskin primary keratinocytes and N/TERT-1 foreskin keratinocytes	Protein knockout	lentiCRISPR v2 and pXPR_011	Lentivirus	SpCas9	NHEJ	Puromycin and blasticidin
	Choi et al., 2019	31319135	HaCaT keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin

(*continued*)

Table 1. Continued

Cell source	Publication	PMID	Cell Types (All Human)	Research Goal	Method of Introduction	Carrier	Cas9 Version	Repair	Selection
	Stump et al., 2020	30972602	HaCaT keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
	Muraguchi et al., 2019	31122679	HaCaT keratinocytes	Protein knockout	TransIT-LT1	Cationic vector	SpCas9	NHEJ	FACS
	Walter et al., 2019	31178865	HaCaT keratinocytes	Protein knockout	Lipofectamine 2000	Cationic vector	SpCas9	NHEJ	FACS
	Hatterschide et al., 2020	32581101	Foreskin primary keratinocytes and N/TERT-1 foreskin keratinocytes	Protein knockout	lentiCRISPR v2 and pXPR_011	Lentivirus	SpCas9	NHEJ	Puromycin and blasticidin
	Casares et al., 2020	31518892	HaCaT keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin
	Gálvez et al., 2020	32637457	Immortalized primary adult keratinocytes	Protein knockout	Electroporation	RNP complex	SpCas9	NHEJ	No
	Enjalbert et al., 2020	32544098	N/TERT foreskin keratinocytes	Protein knockout	FuGene 6 and HiperFect	Cationic vector	SpCas9	NHEJ	FACS
	Kocher et al., 2020	32142798	Immortalized adult primary keratinocytes and RDEB primary keratinocytes	Gene activation and protein knockout	Electroporation	RNP complex	SpCas9	NHEJ	None
	Dabelsteen et al., 2020	32710848	N/TERT foreskin keratinocytes	Protein knockout	lentiCRISPR v2	Lentivirus	SpCas9	NHEJ	Puromycin and blasticidin
	Imahorn et al., 2020	32917957	Immortalized epidermolytic ichthyosis keratinocytes	Protein knockout	Xfect	Cationic vector	SpCas9	NHEJ	FACS
	James et al., 2020	32938703	N/TERT foreskin keratinocytes	Protein knockout	Calcium phosphate transfection	Plasmid vector	SpCas9	NHEJ	Puromycin
	Sobiak and Leśniak, 2020	33297464	HaCaT keratinocytes	Protein knockout	Lipofectamine 3000	Cationic vector	SpCas9	NHEJ	FACS
	Bonafont et al., 2021	33609734	Immortalized adult primary keratinocytes	Protein knockout	Electroporation	RNP complex	SpCas9	NHEJ	None
	Abboodi et al., 2021	33321328	HPV16-transformed foreskin primary keratinocytes	Protein knockout	Lipofectamine 3000	Cationic vector	SpCas9	NHEJ	FACS
	Wanuske et al., 2021	33354837	HaCaT keratinocytes	Protein knockout	Lipofectamine 2000	Cationic vector	SpCas9	NHEJ	FACS
	O'Keeffe Ahern et al., 2021	34363036	Immortalized primary adult keratinocytes	Protein knockout	Lipofectamine 3000	RNP complex	SpCas9	NHEJ	FACS
	Evrard et al., 2021	n/a	N/TERT foreskin keratinocytes	Protein knockout	Electroporation	RNP complex	SpCas9	NHEJ	None
	Kocher et al., 2021	34458008	Immortalized RDEB primary keratinocytes and fibroblasts	Gene correction	Electroporation	RNP complex	SpCas9 and Cas9n	HDR	None
iPSC	Sebastiano et al., 2014	25429056	Induced pluripotent stem cell–derived keratinocytes	Gene correction	Electroporation	Plasmid vector	SpCas9	HDR	Geneticin and ganciclovir
	Webber et al., 2016	28250968	Induced pluripotent stem cells	Gene correction	Electroporation	Plasmid vector	hCas9	HDR	Puromycin
	Shinkuma et al., 2016	27143720	Induced pluripotent stem cells	Gene correction	Electroporation	Plasmid vector	SpCas9	NHEJ	FACS
	Jacków et al., 2019	31818947	Induced pluripotent stem cells	Gene correction	Electroporation	RNP complex	SpCas9	HDR	FACS
	Itoh et al., 2020	32376152	Induced pluripotent stem cells	Gene correction	Electroporation	RNP complex	SpCas9	HDR	Puromycin

Abbreviations: Cas9n, Cas9 nickase; hCas9, human codon optimized Cas9; HDR, homology-directed repair; IDLV, integrase-deficient lentiviral particles; iPSC, induced pluripotent stem cell; JEB, junctional epidermolysis bullosa; NHEJ, nonhomologous end joining; PMID, PubMed identifier; RDEB, recessive dystrophic epidermolysis bullosa; RNP, Ribonucleoprotein; SpCas9, *Streptococcus pyogenes* Cas9.

vector carrying a wild-type *LAMB3* donor template flanked by homology arms (Benati et al., 2018). These elegant studies illustrate that CRISPR-Cas9 can be utilized for the restoration of protein expression in patient-derived KCs through highly specific approaches, for example, through the incorporation of a donor oligonucleotide by HDR or by the use of dual sgRNA to remove a specific DNA sequence to correct for frameshift mutations. In addition, these studies show that gene-corrected, patient-derived KCs generated are usually of high quality in terms of skin-equivalent generation and suitable for grafting onto immunodeficient mice. In principle, that would make them good candidates for ex vivo gene and

cell therapy, as showcased by Hirsch et al. (2017) in the first ever total body transplantation with autologous cells that were corrected and expanded ex vivo.

Human-immortalized KCs as alternative cell source

Human primary KCs in epidermal equivalent culture models represent the in vivo epidermis quite well. However, human donor skin is not always available, primary KCs isolation is time consuming, and primary KCs have a short in vitro lifespan. This conflicts with the extensive culture protocols and serial passaging that are necessary for genome-editing strategies. Therefore, many researchers make use of immortalized KCs in studies that are usually aimed at (i) gene and protein function by full knock out (Abboodi et al., 2021), (ii) the biological consequence of a knock out on cell function or during therapeutic conditions (Abboodi et al., 2021; Casares et al., 2020; Choi et al., 2019; Dahlhoff et al., 2017; Hatterschide et al., 2019, 2020; James et al., 2019; Swindell et al., 2018; Trothe et al., 2018), (iii) validation of therapeutic target (Abboodi et al., 2021; Liu et al., 2016), or (iv) generating disease model cell lines (Enjalbert et al., 2020; Sarkar et al., 2018).

Immortalized KCs, such as the spontaneously immortalized HaCaT KCs, the N/TERT-1, and N/TERT-2G KCs, or the less used HPV16-induced immortalized KCs do not have these limitations and thus provide an alternative unlimited cell source (Boelsma et al., 1999; Smits et al., 2017). Therefore, most studies using CRISPR-Cas9 in human KCs have been performed in either of the immortalized KC cell lines (Figure 1c and Table 1). Although multiple cell sources are available, they are not equally comparable with primary KCs and are not necessarily similarly suited for genomic engineering procedures. The HaCaT KCs are frequently used as a model for KCs in vitro as both monolayer and human skin equivalents (Schoop et al., 1999). However, epidermal stratification is abnormal, aberrant epidermal differentiation protein expression is observed, and a stratum corneum is often lacking. Another drawback is that HaCaT cells show aneuploidy. Taken together, this makes HaCaT KCs less suitable for genome editing and studying epidermal differentiation. The N/TERT-1 and N/TERT-2G KC cell lines were immortalized by the introduction of the hTERT gene and by spontaneous loss of the pRB/p16INK4A cell cycle control mechanism (Dickson et al., 2000). The N/TERT KC cell lines are (largely) diploid (N/TERT-1: 47, XY + 20, N/TERT-2G: 46, XY) and show similar differentiation characteristics to those of human primary KCs (Smits et al., 2017), which renders them more suitable for genomic intervention tools such as CRISPR-Cas9. Immortalized KCs are well-suited for fundamental studies into protein function, possible therapeutic targets, or disease modeling studies but are not applicable for in vivo treatment purposes. In contrast, KCs derived from induced pluripotent stem cells (iPSCs) would be more suitable with regard to regenerative medicine.

KCs derived from CRISPR-Cas9–edited iPSCs

Human pluripotent stem cells (hPSCs) and iPSCs offer great promise in regenerative medicine both for disease modeling and for tissue regeneration because they can proliferate indefinitely and can be differentiated to almost any cell type in the human body (Yamanaka and Blau, 2010). Owing to their unlimited proliferation capacity (Takahashi and Yamanaka, 2006), hPSCs and iPSCs have an apparent advantage over other somatic cells or even adult stem cells in genomic-editing studies using CRISPR-Cas9, especially when clonal selection is necessary. Numerous studies reported such strategies to obtain genome-edited cells from tissues that are normally not easily retrievable (Hendriks et al., 2020; Hockemeyer and Jaenisch, 2016). In dermatological research, most studies are on iPSCs derived from patients with EB. For example, iPSCs were generated from fibroblasts derived from a patient with dominant dystrophic EB carrying a heterozygous *COL7A1* mutation. Subsequently, plasmids carrying Cas9 and mutation-site–specific sgRNAs were transfected into these iPSCs before positive selection by flow cytometry. The mutation-site–specific sgRNAs ensured that the correction of the genetic sequence occurred only on the mutated allele but not on the wild type (Shinkuma et al., 2016). Others show the correction of the *COL7A1* gene in RDEB iPSCs by adeno-associated genome editing (Sebastiano et al., 2014) through the introduction of three plasmids encoding Cas9, gRNA, and donor-repair template (Webber et al., 2016) or through electroporation with sgRNA/Cas9 RNP complexes (Jackόw et al., 2019). Induced KCs (iKCs) derived from gene-corrected iPSCs were grafted onto immunodeficient mice, and 2 months after grafting, a normal expression of COL7A1 is shown (Jackόw et al., 2019). Although the generation of genome-edited iPSCs is relatively easy, differentiation from iPSC toward iKC, especially for resembling primary KCs, is less straightforward (Kogut et al., 2014; Sah et al., 2021; Soares and Zhou, 2020). In addition, iPSC-derived KCs are often immature, compared with primary KCs derived from the skin, which is a common feature of many iPSC-derived cells (Friedman et al., 2018; Soares et al., 2019). Although the traditional air–liquid interface cultures are challenging in iPSC-derived cells, other options are available. Groundbreaking work has shown a human iPSC–based organoid culture system in which skin appendages (e.g., hair follicles and sebaceous glands) are present (Lee et al., 2020). Organoids as such would be suitable to study aspects that are impossible to study in traditional skin equivalents, such as (early) developmental processes. Empowered by CRISPR-Cas9 genomic engineering and analysis techniques at single-cell resolution, these organoid cultures are highly promising options for future research into the skin.

Future perspective for the use of CRISPR-Cas9 in experimental dermatology

To date, no clinical experiments have been performed or are registered using CRISPR-Cas9 in primary KCs to treat skin disorders, although CRISPR-Cas9–based in vivo experiments have been reported in murine models. For example, mouse tail skin was successfully electroporated with DNA plasmids (encoding gRNAs and Cas9) and RNP complexes of synthetic Cas9 and in vitro transcribed sgRNAs (Wu et al., 2017). In 2017, Hirsch et al. (2017) experimentally treated a patient with JEB with a homozygous mutation in the *LAMB3* gene, which owing to the blistering and infections had lost over

80% of his epidermis. Although this is a great example of gene therapy, it was not CRISPR-Cas9 based but was through ex vivo gene replacement by viral transduction of *LAMB3* cDNA.

Conclusion and future directions

Before in vivo CRISPR-Cas9 can be considered in clinical practice, many improvements on CRISPR-Cas9 machinery, that is, component stability, in vivo delivery, editing accuracy, nonspecific and unintended off-target effects, and control of cellular repair mechanisms are necessary (Li et al., 2018). In addition, Cas9 has been reported to elicit immune responses in mice (Chew et al., 2016; Wang et al., 2015) and humans (Simhadri et al., 2018; Wagner et al., 2019), posing a challenge for CRISPR-Cas9–based genomic engineering (Crudele and Chamberlain, 2018). Nevertheless, the impact of this immunological challenge needs to be studied in immunocompetent (humanized) animal models to assess the potential strategies to minimize the impact of anti-Cas9 antibodies and T cells. Until then, realistic and important goals for CRISPR-Cas9 implementation are to further develop in vitro human disease models to benefit preclinical research, therapeutic target discovery, and drug screening.

Monogenetic disorders of the epidermis can be modeled, and the effects of therapies can be studied extensively without the need for primary KCs, patient biopsies, or animal models. Besides KCs, other skin cell types—such as fibroblasts—are of interest too. Research on dystrophic EB pathogenesis indicated that both KCs and fibroblasts are responsible for the expression of COL7 (*COL7A1*), where the contribution of fibroblasts overrules that of KCs (Goto et al., 2006). Fibroblasts are considered a more robust and easier to culture type of cells than KCs, which renders them suitable for prolonged culturing and genomic engineering (Chen and Woodley, 2006) and a potential target cell type for gene and cell therapy in dystrophic EB (Izmiryan et al., 2018; Jackόw et al., 2016; Kocher et al., 2021; Takashima et al., 2019; Webber et al., 2016). As this field of research expands, lessons can be taken from experimental approaches that were successful in epidermal KCs and applied to dermal fibroblasts and vice versa.

Nonspecific endonuclease activity can result in off-target unintended genomic alterations. Ever since the first application of CRISPR-Cas9 in mammalian cells, progress has been made to mitigate the incidence of off-target DNA cleavage by nonspecific endonuclease activity resulting in off-target unintended genomic alterations, as reviewed recently (Naeem et al., 2020). These strategies range from but are not limited to modification of gRNA, modification of Cas9 (e.g., deactivated Cas9 [dCas9], Cas9 nickase [Cas9n], high-fidelity Cas9), fine-tuning delivery methodology, application of base editors (dCas9 combined with deaminase and gRNA), and application of prime editing (Cas9n combined with reverse transcriptase). Therefore, besides selecting editing strategies on the basis of maximizing editing efficiencies and cell viability, different options are now available to minimize off-target risks. These should be taken into consideration depending on which safety measures are applicable for the purpose of genomic engineering.

Besides investing in methodological improvements using currently available (immortalized) KCs (e.g., target DNA site selection, sgRNA design and delivery methods, off-target DNA cleavage, NHEJ and HDR incidence and efficiency, and Cas9 activity), efforts should also be directed to the generation of new skin cell sources to increase experimental diversity and account for population, sex, and age differences. Having CRISPR-Cas9 technology at hand, more complex, multicellular, immunocompetent, and vascularized organotypic skin models with higher throughput can be developed. These innovations will further propel the implementation and acceptance of organotypic human skin models as excellent alternatives or superior experimental models to the traditional use of animals in biomedical research.

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CONFLICT OF INTEREST

The authors state no conflicts of interest.

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