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14. ABSTRACT The proposal develops a stem-cell based therapy for recessive dystrophic epidermolysis bullosa (RDEB), which is one of the most severe forms of epidermolysis bullosa (EB), a group of rare inherited skin blistering diseases. To accomplish this goal, we have utilized genetic correction of patient-specific induced pluripotent stem cells (iPSCs) followed by the differentiation of these corrected iPSCs into epidermal cells and fibroblasts for the generation of composite full thickness skin grafts for transplantation. During the current award, we validated the reproducibility of our combined gene editing and reprogramming approach. We also produced and characterized genetically corrected RDEB iPSCs that we will use to continue the generation of IND-enabling data. We developed a cGMP-compliant procedure for the generation of these iPSCs in the Gates Biomanufacturing Facility (GBF). In addition, we developed a cGMP-compatible procedure for enriching keratinocytes derived from iPSCs (iKCs) using a cGMP-compliant CliniMACS Prodigy enrichment approach. We did face a problem of inconsistent engraftment of iKCs and iPSC-derived fibroblasts (iFBs). For this reason, we modified our protocols for the derivation of iKCs and iFBs. We now derive these cells from hair-bearing human skin organoids. We can now consistently generate hair bearing skin organoids from all our genetically corrected RDEB iPSCs. We also found that iKCs and iFBs isolated from these organoids show more consistent functionality as compared to iKCs and iFBs generated using our conventional monolayer protocol. The organoid-based differentiation protocol will now allow us to generate both genetically corrected RDEB iKCs and iFBs in one differentiation procedure followed by CliniMACS Prodigy enrichment using cell type specific surface markers. This will simplify and reduce the cost of manufacturing our stem cell therapy for EB. The data generated with the current support and other sources allowed us to successfully compete for a FY2022 PRMRP Expansion Award (PR222029) and receive the funding to continue our efforts toward the clinical translation of our iPSC-based therapy for EB.					
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1. INTRODUCTION:

This application proposes to advance our previously developed induced pluripotent stem cell (iPSC)-based therapy for recessive dystrophic epidermolysis bullosa (RDEB) into the clinic by adapting the production of genetically corrected patient-specific iPSC-derived epidermal sheets and composite full-thickness skin grafts to current Good Manufacturing Practice (cGMP) standards and by generating a set of preclinical data for submission of an Investigational New Drug (IND) application. Therefore, this application directly addresses the FY17 PRMRP Topic Area “**Epidermolysis Bullosa**”. This application is also relevant to one of the Areas of Encouragement identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (as indicated in Appendix 2 of the Program Announcement) since, if successful, the study will result in the approval of a Phase I Clinical trial for a product that may enhance wound healing in inherited epidermolysis bullosa (EB). There are significant procedural differences when a cell therapy product is manufactured under general laboratory settings vs when the same product is manufactured under cGMP-compliant conditions in a cGMP facility. In this proposal, we will transfer the technologies that we have developed using our previous awards from the federal government and private foundations to a product development laboratory at a cGMP-compliant facility. We will also perform a pilot small-scale cGMP production run of genetically corrected RDEB iPSCs and epidermal progenitors derived from genetically corrected RDEB iPSCs. In addition, we will develop a composite skin graft consisting of both genetically corrected iPSC-derived keratinocytes and fibroblasts as an alternative to genetically corrected iPSC-derived epidermal sheets for the treatment of RDEB. If successful and proven to be safe in a clinical trial for EB, the iPSC-based therapy could then be easily expanded to monogenic diseases affecting internal organs, where the difficulty in monitoring adverse effects of an iPSC-based therapy would make them unlikely first targets. The iPSC-based therapy may potentially be applied to military personnel who develop severe blistering following exposure to vesicants, or who suffer from burns over a large portion of their body. In addition, stem-cell based therapies could also be used to accelerate wound repair in military personnel who experience acute injuries, or in older veterans with chronic wounds.

2. KEYWORDS:

Epidermolysis Bullosa (EB); Recessive Dystrophic EB (RDEB), Induced Pluripotent Stem Cells (iPSC); stem cell-based therapy; current Good Manufacturing Practice (cGMP) standards.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: To perform a pilot cGMP-compliant production of genetically corrected RDEB iPSCs.

<u>Task 1.1</u> To verify the reproducibility of simultaneous reprogramming and gene editing on cells isolated from three independent patients sharing the <i>COL7A1</i> ^{c.7485+G>A} mutation (3 human subjects)	100% completed
<u>Task 1.2</u> To validate the absence of off-target events post-correction	100% completed
<u>Task 1.3</u> To validate custom-made antibodies to quantify the level of WT Col 7 post- <i>COL7A1</i> ^{c.7485+G>A} correction	100% completed
<u>Task 1.4</u> To examine the functionality of corrected RDEB iPSCs	100% completed
<u>Task 1.5</u> To transfer the protocols to a product development laboratory at a cGMP-compliant facility, perform optimizations and prepare documentation/batch record	100% completed
<u>Task 1.6</u> To implement a pilot small-scale cGMP-compliant run of the protocol for the generation of a MCB of genetically corrected iPSCs at a cGMP-compliant facility	100% completed
Milestone(s) Achieved: Successful pilot cGMP production of genetically corrected RDEB iPSCs	Completed
Local IRB/IACUC Approval	Completed
Milestone Achieved: HRPO/ACURO Approval	Completed

Aim 2: To perform a pilot cGMP-compliant production of epidermal progenitors from genetically corrected iPSCs..

<u>Task 2.1</u> To confirm reproducibility of keratinocyte differentiation protocol on iPSCs with the corrected <i>COL7A1</i> ^{c.7485+G>A} mutation (3 human subjects)	100% completed
<u>Task 2.2</u> To transfer the protocol for the generation of epidermal progenitors to a product development laboratory at a cGMP-compliant facility, perform optimizations and prepare documentation/batch record	100% completed
<u>Task 2.3</u> To implement a pilot small-scale cGMP-compliant run of the protocol for the generation and characterization of genetically corrected iPSC-derived epidermal progenitors at a cGMP-compliant facility	100% completed
Milestone(s) Achieved: Successful pilot cGMP production of genetically corrected iPSC-derived epidermal progenitors	Completed

Aim 3: To develop a cGMP-compatible protocol for the generation of a composite skin graft and to generate IND-enabling safety and efficacy data for the FDA

Task 3.1 To optimize a protocol for the differentiation of iPSCs into a fibroblast lineage	100% completed
Task 3.2 To examine the functionality of iPSC-derived fibroblasts (12 mice for xenotransplantation)	100% completed
Task 3.3 To develop a cGMP-compatible protocol for the differentiation of <i>COL7A1</i> ^{c.7485+G>A} corrected iPSCs into fibroblasts	100% completed
Task 3.4 To generate a composite graft using genetically corrected iPSC-derived keratinocytes and fibroblasts in organotypic cultures and verify type VII collagen deposition	100% completed
Task 3.5 To assess wound closure by iPSC-derived composite grafts in immunocompromised mice	100% completed
Task 3.6 To assess tumorigenicity and the presence of residual iPSCs in the composite grafts	100% completed
Milestone(s) Achieved: Generation of Pre-IND-enabling safety and efficacy data for composite grafts using cGMP-compatible protocols	Completed

What was accomplished under these goals?

*Task 1.1 To verify the reproducibility of simultaneous reprogramming and gene editing on cells isolated from three independent patients sharing the *COL7A1*^{c.7485+G>A} mutation (3 human subjects).*

We obtained a determination from the HRPO that our study is exempt from being considered human subjects research. The decision was made on November 6, 2019. This allowed us to focus our studies on previously collected de-identified specimens from three RDEB patients (CO1, CO2 and CO4). Two female patients were homozygous for the *COL7A1*^{c.7485+G>A} mutation, and one male RDEB patient was heterozygous for the *COL7A1*^{c.7485+G>A} mutation. These lines were used to validate the reproducibility of our simultaneous gene editing and reprogramming protocol.

For the *COL7A1* correction strategy, a gRNA was designed to target Cas9 as close as possible to the c.7485+5G>A mutation, thereby facilitating the correction of the *COL7A1*^{c.7485+G>A} mutation via homology-directed repair (HDR) using a 80bp single-stranded donor oligonucleotides (ssODN). Two different types of ssODN donors were also assessed: an unmodified ssODN donor and a ssODN donor carrying three silent SNPs to increase the efficiency of the correction. Briefly, the patient fibroblasts were plated at a low density and transfected with mod-mRNA encoding Cas9 together with gRNA and ssODN using a lipofectamine-based

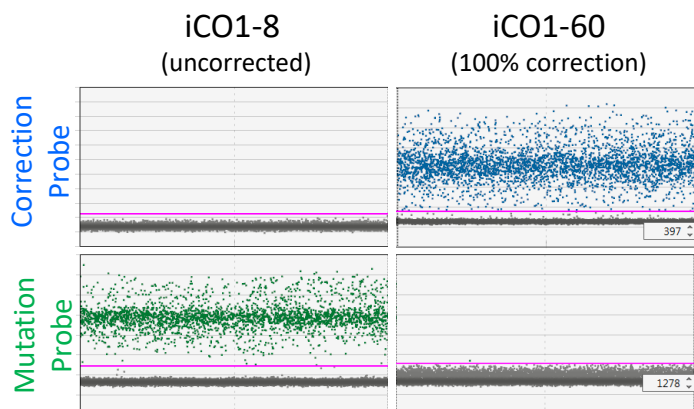


Fig. 1. Successful correction of the *COL7A1*^{c.7485+G>A} mutation on both *COL7A1* alleles in CO1 RDEB fibroblast line as detected by ddPCR. After combined reprogramming and gene editing, colonies were manually isolated, clonally expanded, and screened by ddPCR. The hydrolysis probe indicated in green detects the c.7485+5G>A mutation, while the probe indicated in blue detects the corrected sequence (including the incorporated 3 silent mutation). The iPSC line iCO1-8 was confirmed to be unmodified, while iCO1-60 showed a complete lack of any *COL7A1* mutation (green) and only the presence of the corrected sequence (blue).

method. After gene editing, the fibroblasts were transfected every-other-day with a cocktail of reprogramming RNAs (total transfection). At the end of reprogramming, iPSC colonies could be manually isolated and clonally expanded to generate isogenic cell lines and screened by ddPCR. **Fig. 1** shows a representative result of one of the correction experiments as determined by ddPCR. In this experiment, we successfully corrected both *COL7A1*^{c.7485+G>A} alleles during our combined gene editing and reprogramming of the homozygous CO1 fibroblasts line. As shown in **Fig. 1**, in our ddPCR assay, the hydrolysis probe indicated in green detects the c.7485+5G>A mutation, while the probe indicated in blue detects the corrected sequence (including the incorporated 3 silent mutation for CO1 and CO4 lines). The iCO1-8 iPSC line was confirmed to be unmodified, while the iCO1-60 iPSC line showed a complete lack of any *COL7A1* mutation (green) and only the presence of the corrected sequence (blue). Furthermore, the corrected count equaled the number of alleles from an unaffected housekeeping gene, indicating correction of both *COL7A1* alleles (not shown).

The successful correction was further confirmed by Sanger sequencing. A representative sequencing analysis for the line where both *COL7A1* alleles are corrected is shown in **Fig. 2**.

As a result, we successfully generated corrected clones from all three RDEB specimens and control uncorrected clones from the same experiment. **Table 1** summarizes our results. The efficiency of correction varied between 2 and 5% across experiments without any significant differences between unmodified ssODN and ssODN carrying silent SNPs. While our goal is to correct at least one mutant *COL7A1* allele, the CO1-60 line appeared to undergo a dual correction in which both *COL7A1* alleles were corrected to a wild type sequence by HDR (see **Fig. 1** and **2**). The analysis of the generated clones for pluripotency and genetic stability is currently in progress. To date, we characterized one corrected CO4 clone, which showed the expression of pluripotency markers and normal karyotyping (**Fig. 3**). One corrected clone from our correction (CO2-6) was shared with the groups at Stanford and Columbia Universities for characterization and differentiation studies as outlined in our original application. The results of our collaboration with Stanford and Columbia Universities have been consolidated in a manuscript that has been submitted to BioRxiv as a preprint. The DOD funding source has been acknowledged. This manuscript is currently undergoing a peer review process. The preprint of this work is included into Appendix 1 (Neumayer et, A scalable, GMP-compatible, autologous organotypic cell therapy for Dystrophic Epidermolysis Bullosa, 2024)

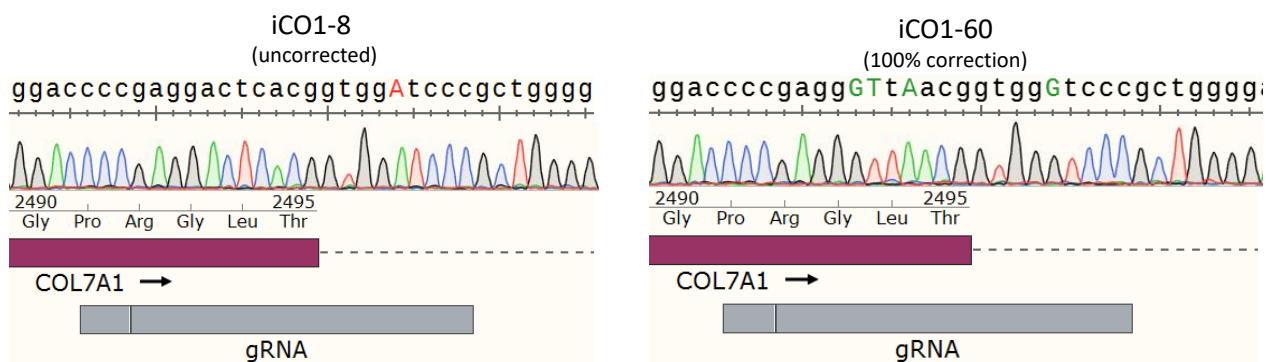
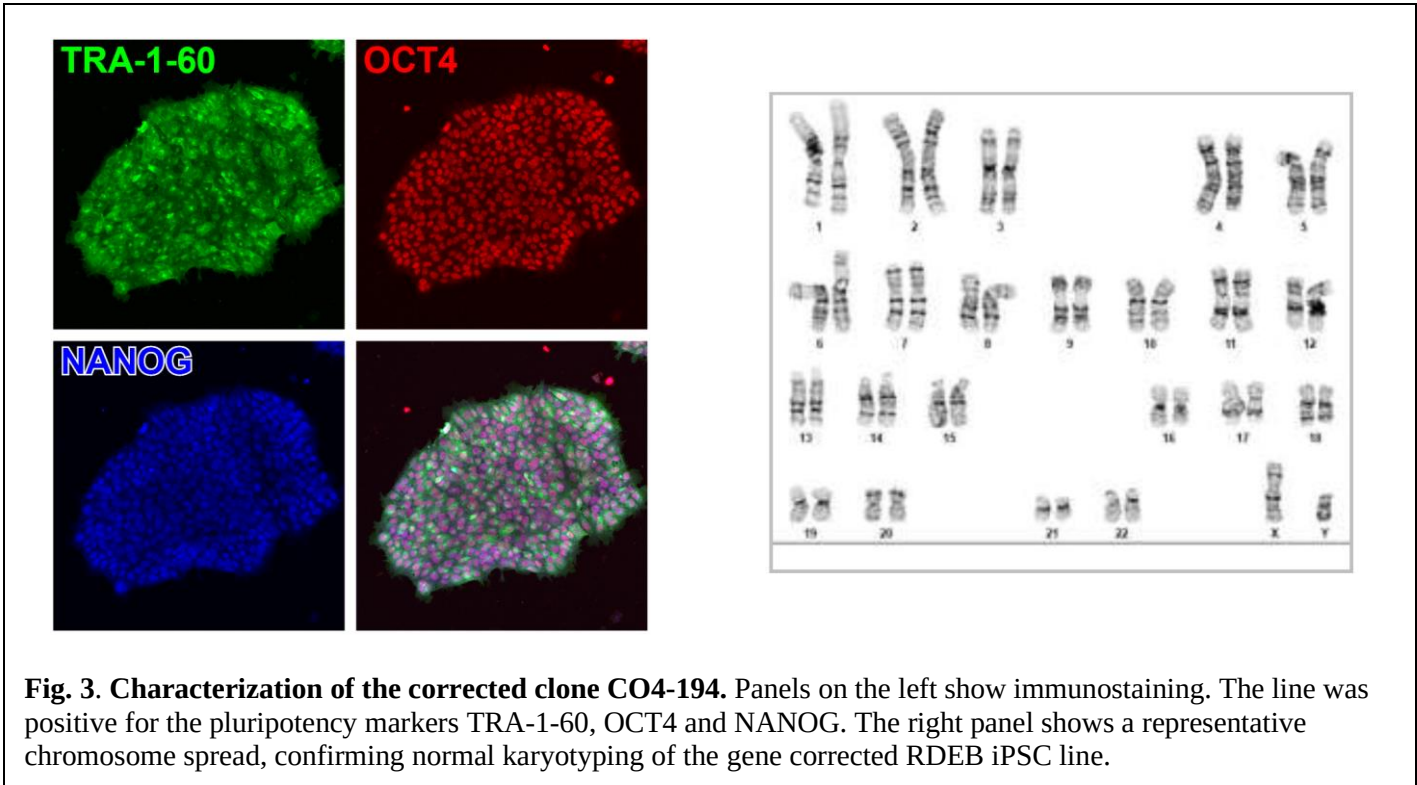


Fig. 2. Sanger sequencing confirmed the successful generation of RDEB iPSC clones with both alleles being corrected. In uncorrected iCO1-8, the *COL7A1*^{c.7485+5} locus was amplified by PCR, Sanger sequenced, and confirmed to harbor the intronic G>A mutation (indicated in red). In iCO1-60, the *COL7A1*^{c.7485+5} locus was amplified by PCR, Sanger sequenced, and confirmed to be corrected in addition to the incorporation of the 3 silent mutations (indicated in green). The lack of any mixed peaks further indicates that both alleles were corrected.

Table 1: Successful generation of gene corrected RDEB iPSC clones from three patient fibroblast lines carrying the c.7485+5G>A mutation

iPSC line	COL7A1 status	Allele 1	Allele 2
CO1-8	RDEB (control)	c.7485+5G>A	c.7485+5G>A
CO1-60	corrected	corrected	corrected
CO2-18	RDEB (control)	c.7485+5G>A	c.7485+5G>A
CO2-6	corrected	corrected	c.7485+5G>A
CO4-219	RDEB (control)	c.7485+5G>A	c.4621delG
CO4-194	corrected	corrected	c.4621delG



Task 1.2 To validate the absence of off-target events post-correction.

We did not identify any off-target modifications in our three corrected RDEB iPSC clones (CO1-60, CO2-6 and CO4-194) by analyzing predicted off-target sites. We have performed CIRCLE Sequencing (CIRCLE-Seq) on RDEB fibroblasts to identify additional potential off target sites. By doing this analysis, we identified 39 sites that can potentially be targeted by the gRNA designed to correct the c.7485+G>A mutation (**Fig. 4**). We used the UCSC chromosome locator (hg38) to find reported off-target sites (**Fig. 5**). To date, we have sequenced 10 top sites that we identified by CIRCLE-Seq in two genetically corrected iPSC lines generated from two different patients and did not find any mutations. This confirms our finding that our combined gene editing and reprogramming protocol is effective and safe.

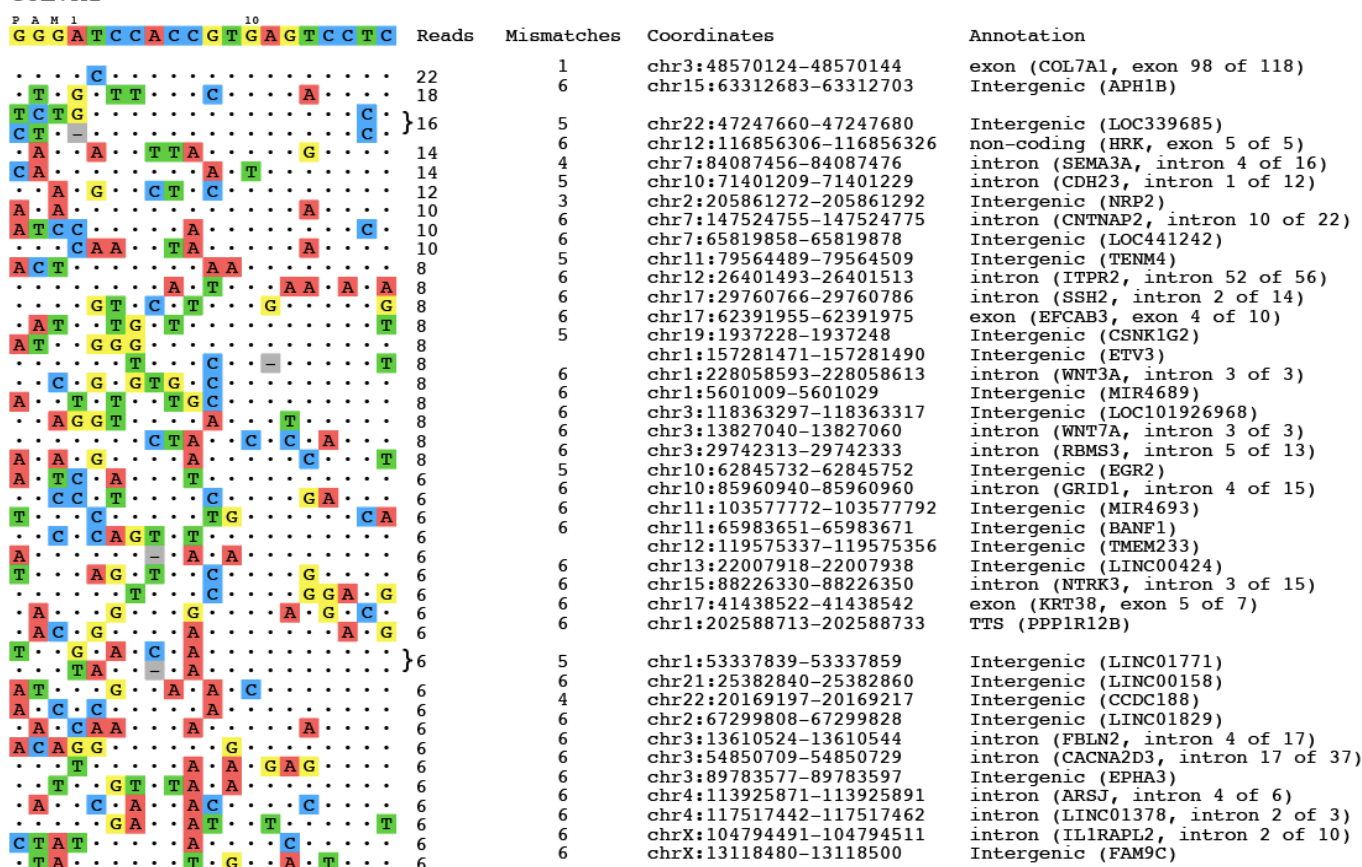


Fig. 4. Identification of Cas9 non-specific cleavage sites in the gene editing strategy to correct the *c.7485+G>A* mutation using CIRCLE-Sequencing. We isolated genomic DNA from the RDEB fibroblast line with the homozygous *c.7485+G>A* mutation. The DNA was sheared and circularized. Circular DNA molecules were then cleaved by Cas9 nuclease and the cleavage sites were detected by paired-end reads high-throughput sequencing. A total of 39 sites have been identified.

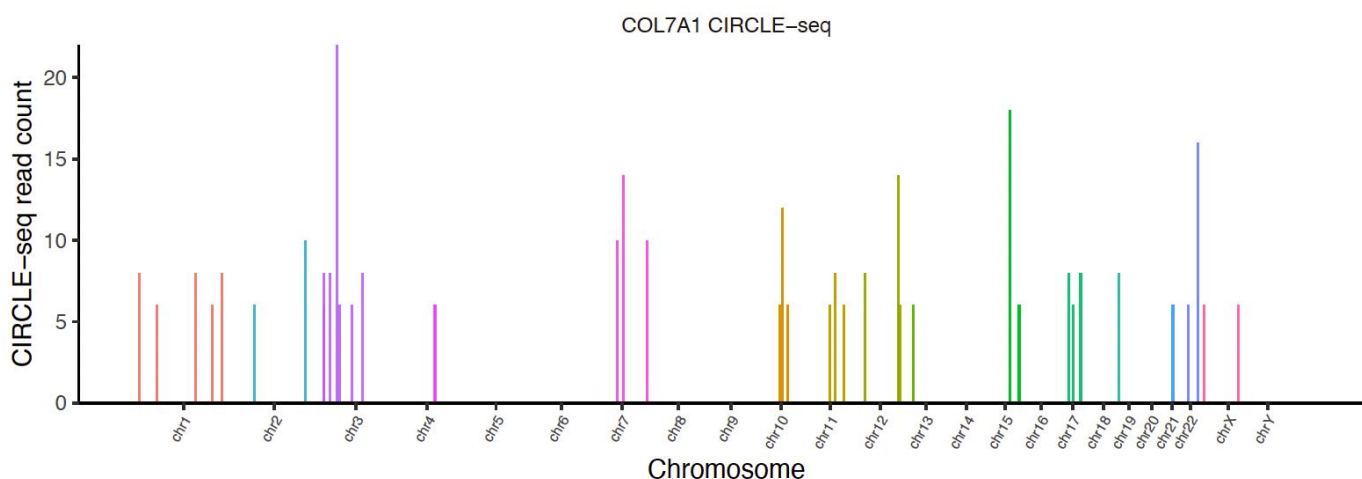


Fig. 5. The distribution of potential off-target sites on chromosomes as determined by CIRCLE-Sequencing.

Since we have multiple patients carrying the Colorado founder mutation, we analyzed the origin of this mutation. This study was important to identify the baseline of mutations in these patients in preparation for our whole genome sequencing analysis. In addition, the study would allow for identifying additional patients suitable for our clinical trial based on the ancestry. The study included several patients with the Colorado mutation, as well as multiple RDEB patients with other recurrent mutations, such as the unique c6527insC mutation (**see below**). We found that the Colorado mutation originated on Native American haplotype with the substantial amount of Sephardic ancestry. These results are summarized in our work published in 2021. The current funding was acknowledged and the paper printout is included in Appendix 2 (**Warshauer et al., Ancestral patterns of recessive dystrophic epidermolysis bullosa mutations in Hispanic populations suggest Sephardic ancestry. *Am J Med Genet A*. 2021 Aug 26;. doi: 10.1002/ajmg.a.62456**).

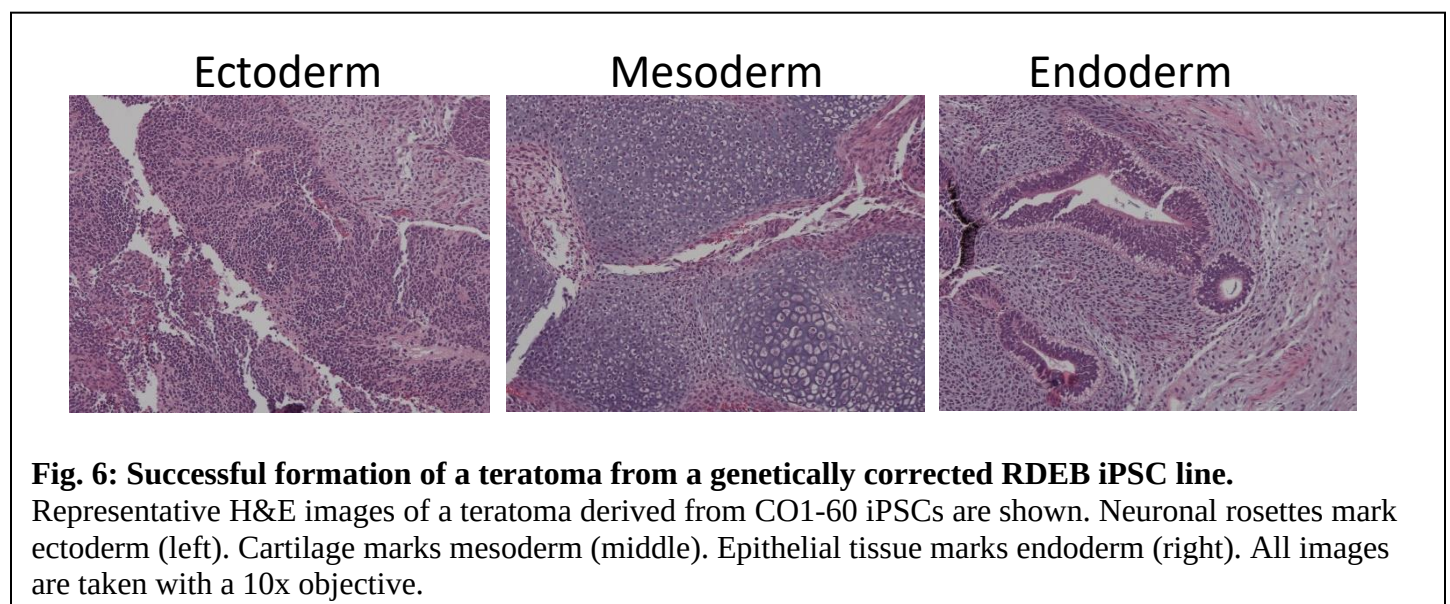
Recently, we have extended our investigation to a global cohort of 126 RDEB individuals with the c.6527insC founder mutation from Spain, France, Argentina, Chile, Colombia, and the USA. Sephardic ancestry was identified at the haplotype spanning the c.6527insC mutation in 85% of the individuals, despite mixed ancestry elsewhere in the genome and no known recent Sephardic ancestry. An age estimation analysis of the c.6527insC mutation was performed and identity-by-descent matching between this RDEB subpopulation and a known crypto-Jewish community in Belmonte, Portugal was ascertained, providing support for crypto-Jewish ancestry in this unique RDEB subpopulation. Thus, we have identified another large cohort of RDEB patients where a single guide RNA could be used to edit the c.6527insC mutation. The current DOD funding was acknowledged in this manuscript which is currently under review and included in Appendix 4 (**Warshauer et al., Secret Sephardic Origins Revealed for the Rare Skin Disorder, Recessive Dystrophic Epidermolysis Bullosa, in Individuals Carrying the Unique c.6527insC Mutation**).

***Task 1.3** To validate custom-made antibodies to quantify the level of WT Col 7 post-COL7A1^{c.7485+G>A} correction.*

We identified a human Col7 antibody that can be used for validating the restoration of COL7A1 expression. See relevant immunofluorescence staining below.

***Task 1.4** To examine the functionality of corrected Col7 in RDEB iPSCs (18 mice for teratoma and 48 mice for xenotransplantation)*

We have successfully obtained teratomas from all genetically corrected iPSCs. We injected 2×10^6 genetically corrected RDEB iPSCs subcutaneously into the flanks of SCID mice. All lines contributed into three germ layers as expected (see **Fig.6**).



All clones have been successfully differentiated into keratinocytes and show the restoration of Col7 expression. See results below in appropriate sections.

***Task 1.5** To transfer the protocols to a product development laboratory at a cGMP-compliant facility, perform optimizations and prepare documentation/batch record.*

We have trained the personnel at the Gates Biomanufacturing Facility (GBF) to perform reprogramming and produce RNA. We performed parallel runs with the GBF and confirmed the reproducibility of our protocols.

***Task 1.6** To implement a pilot small-scale cGMP-compliant run of the protocol for the generation of a MCB of genetically corrected iPSCs at a cGMP-compliant facility.*

The GBF successfully produced modified mRNA reagents and reprogrammed cells in their facility.

***Task 2.1** To confirm reproducibility of the keratinocyte differentiation protocol on iPSCs with the corrected COL7A1^{c.7485+G>A} mutation (3 human subjects).*

We successfully developed a protocol for the differentiation of genetically corrected iPSCs into keratinocytes. We have differentiated all three genetically corrected RDEB iPSC lines into keratinocytes and assessed the formation of the basement membrane in a 3D organotypic cultures as previously published using devitalized dermis (Sebastiano et al. 2013). However, as mentioned in the previous report, we have noticed that occasionally the generation of 3D skin fails. The main reasons for the failure include uneven thickness of the devitalized dermis, infection, over-drying of the prep at the air-liquid level, etc. To improve the consistency of the generation of 3D skin equivalents, we have replaced devitalized dermis with an FDA approved Alloderm.

In addition, as stated in the annual report from 2019, we have performed a more extensive screening of keratinocyte markers suitable for the purification using MACS and found that another integrin –CD104 (integrin $\beta 4$) may be more selective for keratinocytes. In addition, we found that undifferentiated iPSCs express integrin $\alpha 6$ at a high level. We have also performed a spiking experiment mixing human keratinocytes (Lonza) with 5% of undifferentiated iPSCs followed by MACS Separation using α -CD49f magnetic beads. As expected, we failed to eliminate undifferentiated iPSCs from the mixture when α -CD49f magnetic beads were used (see

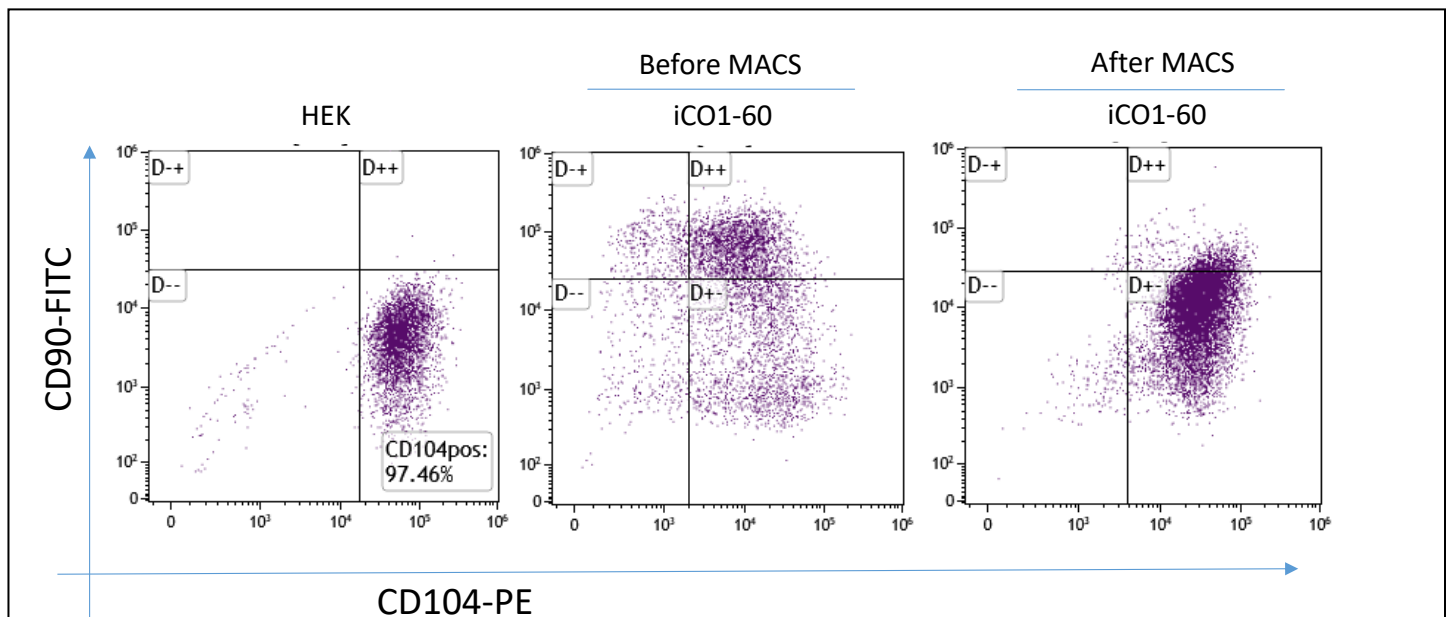
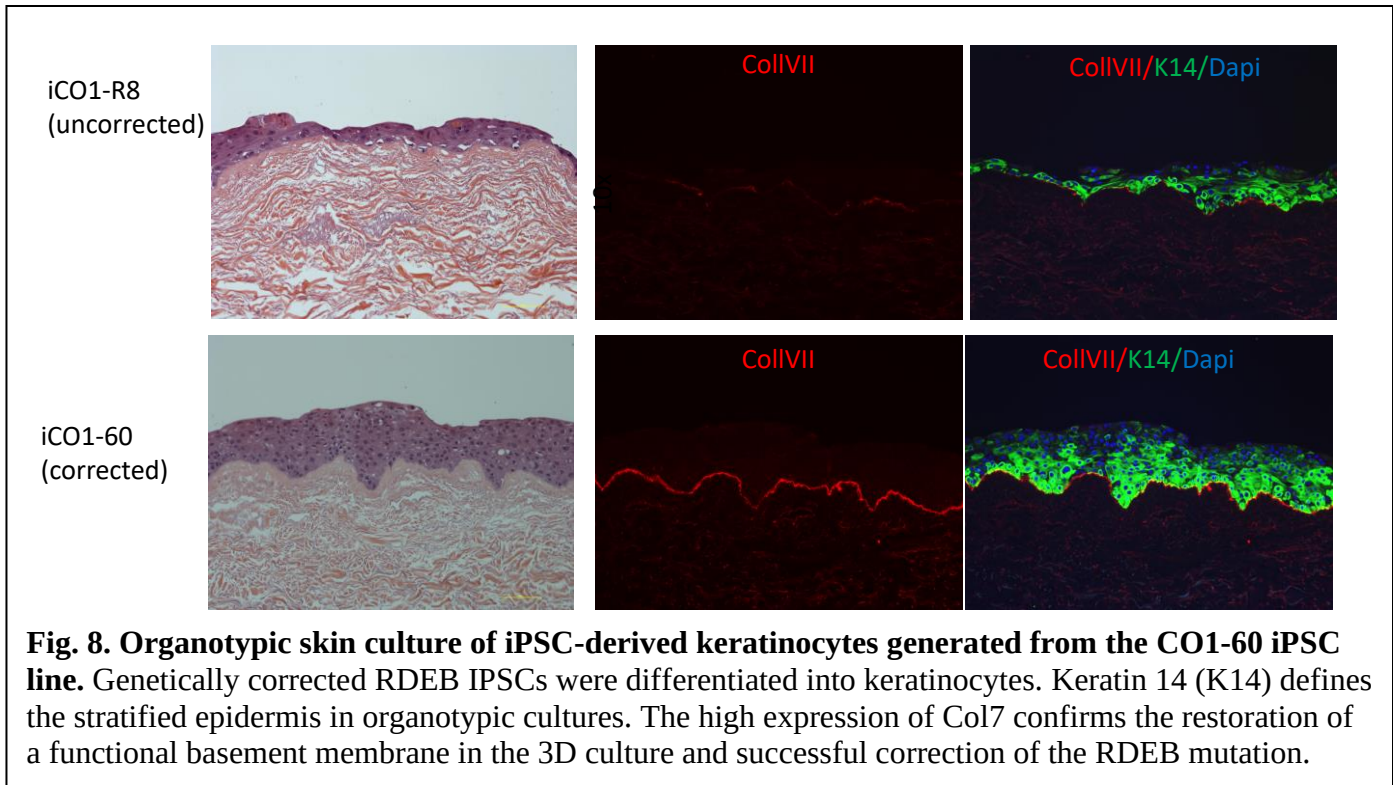


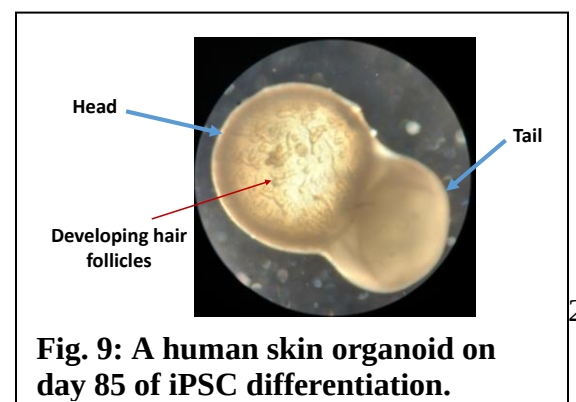
Fig. 7. Enrichment for iPSC-derived keratinocytes using α -CD104 magnetic beads. Genetically corrected RDEB iCO1-60 iPSCs were differentiated into keratinocytes (iKCs) using 1 μ M retinoic acid and 5 ng/ μ L BMP4. At day 45, iKCs were harvested (middle panel) and enriched for CD104 using MACS (right panel). The marker expression profile in the resulting culture was similar to that of healthy keratinocytes (HEK, left panel). The cells expressed high CD104 and low CD-90 levels.

our annual report from 2019 for details). Therefore, another explanation for the inconsistent generation of 3D organotypic cultures could be the use of incorrect selection markers for keratinocyte enrichment. We have repeated our differentiation protocol with α -CD104 magnetic beads for selection of iPSC-derived keratinocytes using all three genetically corrected iPSC lines. As expected, the selection with CD104 resulted in a high-efficiency enrichment (**Fig. 7**).

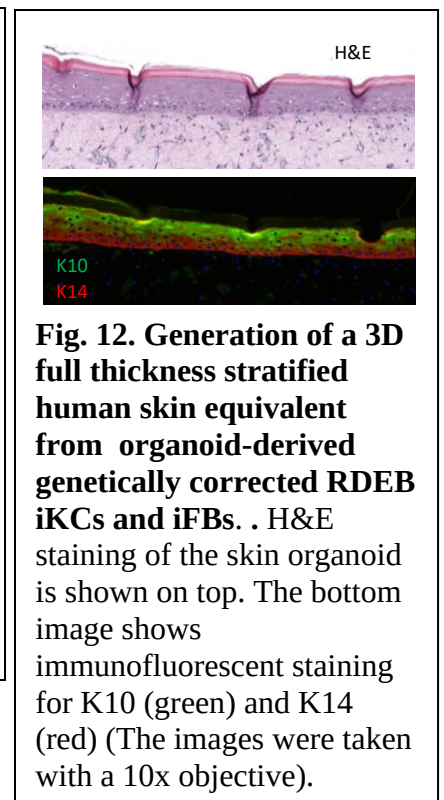
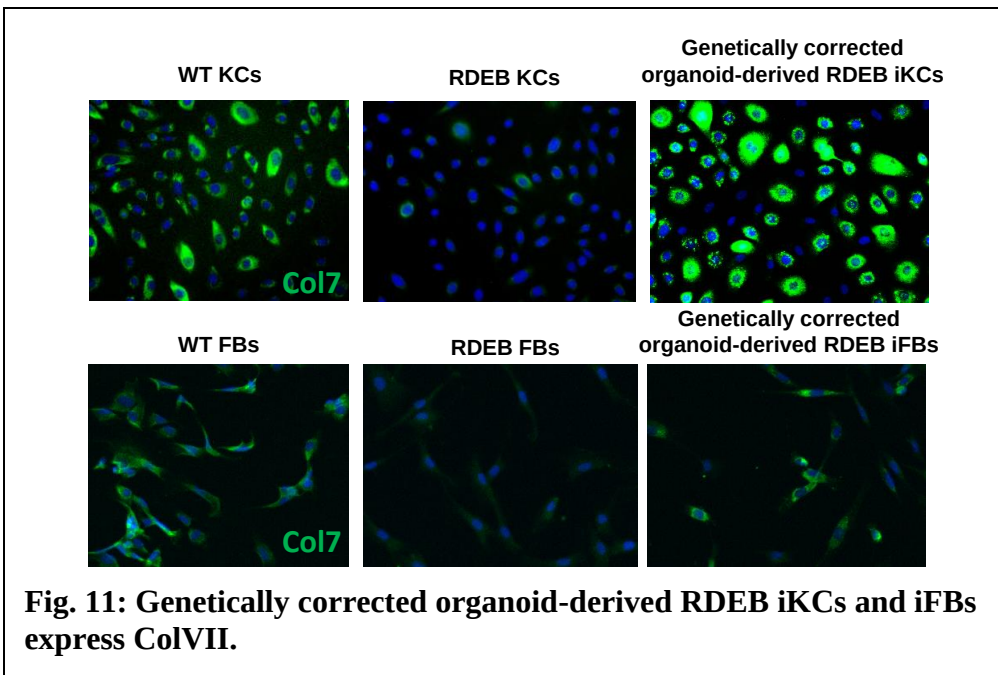
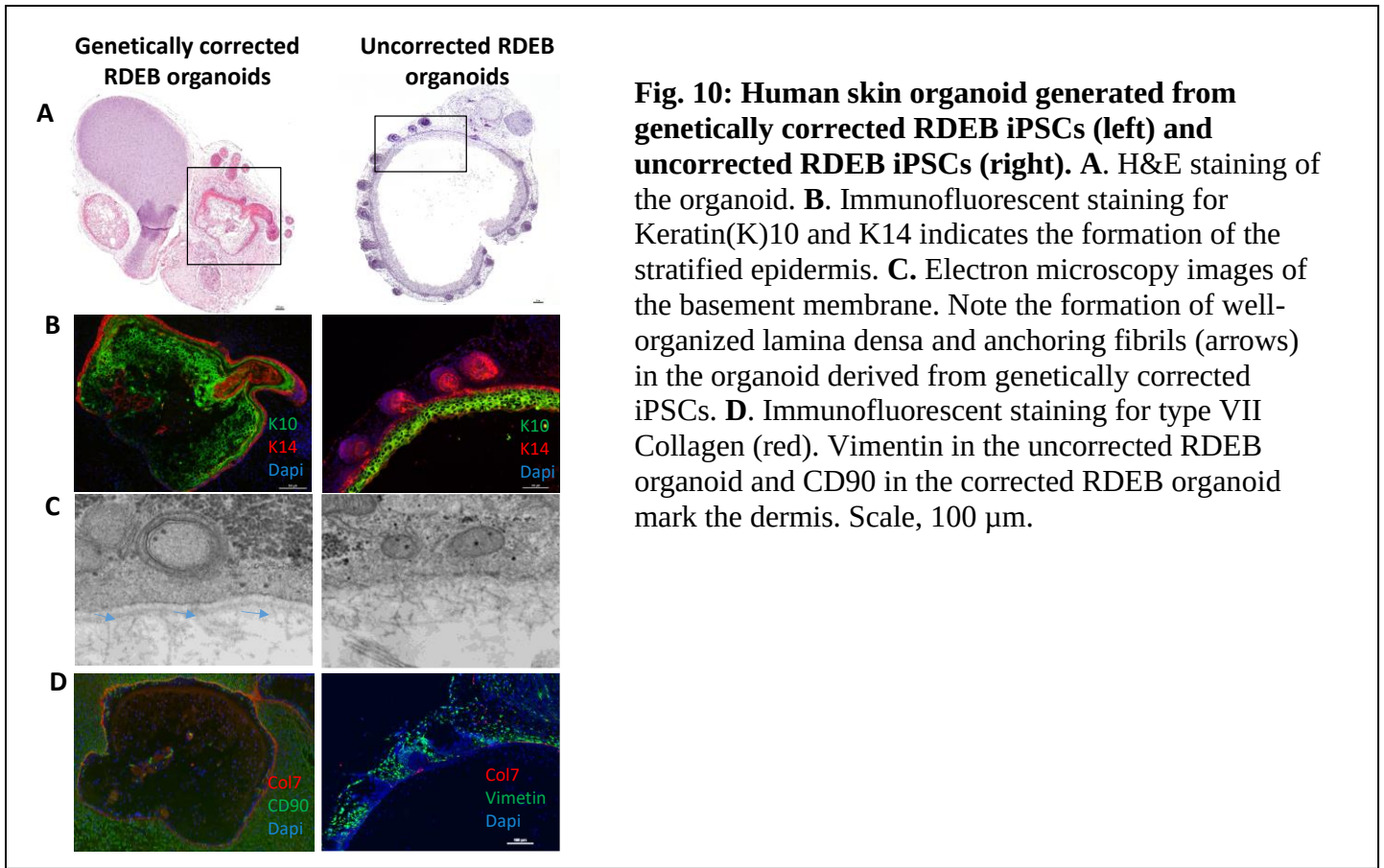
Using the improved protocol and Alloderm-based organotypic cultures, we can now consistently generate epidermis from genetically corrected RDEB iPSC-derived keratinocytes. In **Fig. 8**, the epidermis generated on Alloderm using genetically corrected iPSC-derived keratinocytes (from iCO1-60 iPSCs) shows a more organized structure and a high level of Col7 expression in the basement membrane than the epidermis generated from uncorrected RDEB iPSCs (iCO1-R8 clone).



Unfortunately, we found that iKCs derived using a monolayer differentiation protocol did not engraft consistently on immunocompromised mice. For this reason, we re-evaluated our differentiation protocols. Given that 2D *in vitro* conditions do not fully recapitulate developmental processes, we assessed whether organoids derived from iPSCs provide conditions that are more natural to induce the differentiation of iPSCs into correct lineages. Using the ongoing DOD award and other awards from the NIH and private foundations, we were able to adapt a protocol for the generation of hair-bearing human skin organoids published by Lee et al (2020). We successfully differentiated all three genetically corrected RDEB clones into skin organoids. During its differentiation, an organoid forms a so-called head (where the skin epidermis and hair follicles are formed) and a tail (a cartilage enriched structure that stabilizes the organoid) (**Fig. 9**). By day 100-120, organoids form a fully developed epidermis, hair follicles and a dermal layer (**Fig. 10A-B**). Importantly, the organoid developed from genetically corrected RDEB iPSCs forms an organized basement membrane (**Fig. 10C**), which stains for Col7 (**Fig. 10D**). This confirms that genetic correction of RDEB iPSCs restores the expression of Col7 in skin cells. We have successfully isolated both genetically corrected iKCs and iFBs from organoids and confirmed that both of these cell types express type VII collagen unlike uncorrected primary RDEB fibroblasts and keratinocytes from the same patient (**Fig. 11**). Both organoid-derived iKCs and iFBs could form 3D skin equivalents *in vitro* (**Fig. 12**). The organoid-based differentiation



protocol will now allow us to generate both genetically corrected RDEB iKCs and iFBs in one differentiation procedure, thus simplifying and reducing the cost of manufacturing our stem cell therapy for EB.



Task 2.2 To transfer the protocol for the generation of epidermal progenitors to a product development laboratory at a cGMP-compliant facility, perform optimizations and prepare documentation/batch records.

We developed a protocol for the purification of authentic iPSC-derived keratinocytes (iKCs) based on the CD104 expression using the CliniMACS Prodigy. After purifying a mix of keratinocytes and mesenchymal stem/stromal cells (MSCs), the target cell population (keratinocytes) was 97.3% double positive for CD49f and CD104. The non-target population (MSCs) was 98.4% positive for CD90 (Fig. 13).

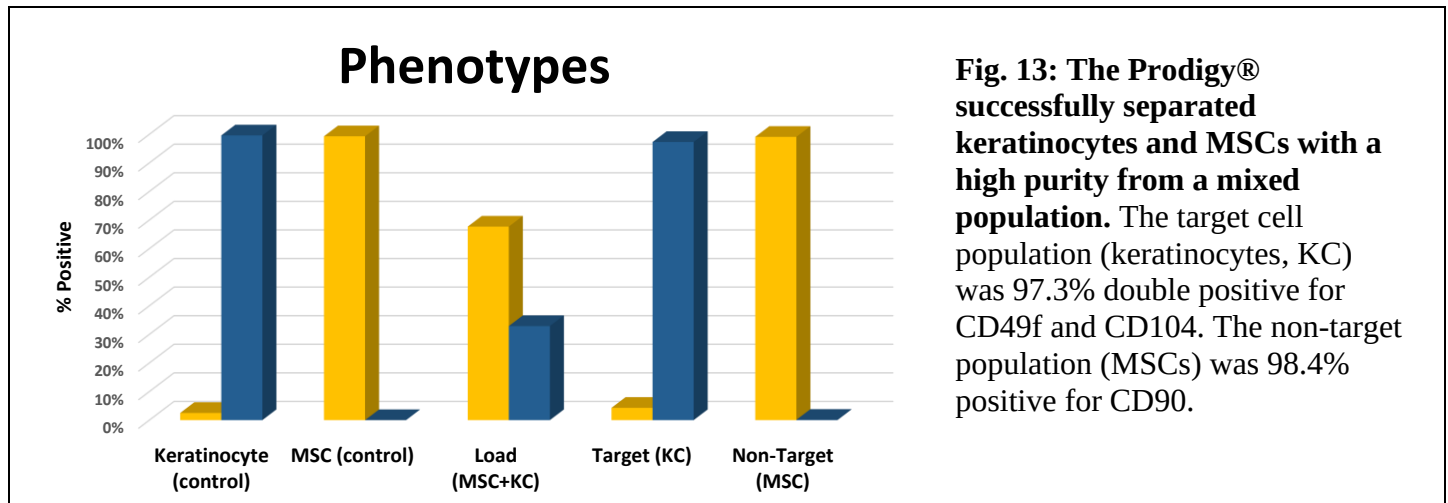


Fig. 13: The Prodigy® successfully separated keratinocytes and MSCs with a high purity from a mixed population. The target cell population (keratinocytes, KC) was 97.3% double positive for CD49f and CD104. The non-target population (MSCs) was 98.4% positive for CD90.

We have also modified our differentiation protocol to produce keratinocytes from skin organoids (see above). We developed cGMP-compatible conditions for culturing these iPSC organoid-derived keratinocytes. We found that the presence of feeder cells is important to promote the functionality of iPSC-derived keratinocytes. We worked with the GBF to develop a protocol for the production of cGMP-compatible feeder layers. After multiple consultations with regulatory consultants, we decided to produce feeder cells from irradiated healthy human neonatal fibroblasts that we can qualify for clinical use. We tested these fibroblasts vs iPSC-derived fibroblasts and found them to be comparable in promoting functionality of iPSC-derived keratinocytes. Given the differences between the monolayer iPSC-differentiation protocol, which unfortunately appeared to be inconsistent for the production of functional keratinocytes, and organoid-based protocols, we used preliminary data generated with this award to apply for another PRMRP expansion award to adapt the production of organoid-derived keratinocytes, fibroblasts and dermal papillae cells to cGMP manufacturing. The new application has been successfully funded (Log# PR222029; PI: Dennis Roop) and will allow us to continue the development of our iPSC-based therapy and the generation of preliminary safety data for the FDA.

Task 2.3 To implement a pilot small-scale cGMP-compliant run of the protocol for the generation and characterization of genetically corrected iPSC-derived epidermal progenitors at a cGMP-compliant facility.

We have already performed one trial run for the production of iPSC-derived keratinocytes from iPSCs using our monolayer differentiation protocol. Therefore, this task has been completed. The adaptation of organoid-based differentiation protocol, which we found to produce better-quality keratinocytes for grafting, will be accomplished using our new award (PR222029).

Task 3.1 To optimize a protocol for the differentiation of iPSCs into a fibroblast lineage.

We have developed optimized the monolayer protocol for the differentiation of iPSCs into fibroblasts and generated iPSC-derived fibroblast-like cells that were negative for pluripotency markers and positive for fibroblasts markers (Fig. 14).

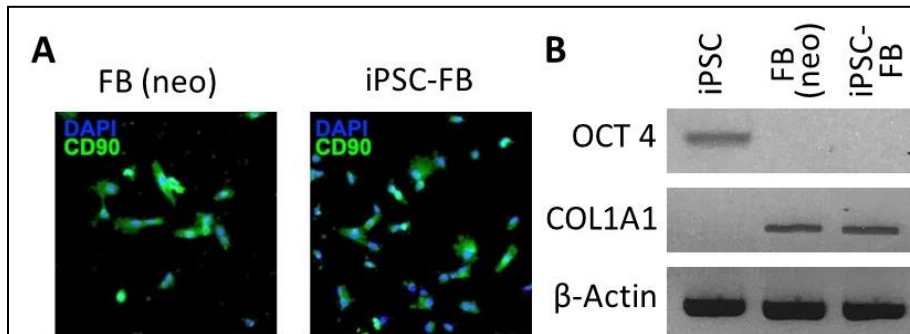


Fig. 14. Fibroblasts derived from iPSCs (iPSC-FB) express appropriate markers as determined by the immunofluorescence staining for CD90 (A) and RT-PCR for Col1 (B). FB (neo) are control neonatal fibroblasts.

Task 3.2 To examine the functionality of iPSC-derived fibroblasts.

Although the fibroblasts that we derived from iPSC using a monolayer protocol expressed the correct markers (Fig. 14), they failed to produce 3D skin equivalents in vitro and engraft onto a mouse in multiple grafting experiments using different grafting techniques. For this reason, we re-evaluated our protocol for the derivation of fibroblasts and found that iPSC-derived skin organoids (Fig. 9 and 10) provide a source of both functional keratinocytes and fibroblasts. We successfully differentiated multiple different iPSC-lines into fibroblasts via skin organoids. All fibroblasts that we obtained could be engrafted using our grafting chamber assay (Fig. 15). Therefore, this task has been accomplished.

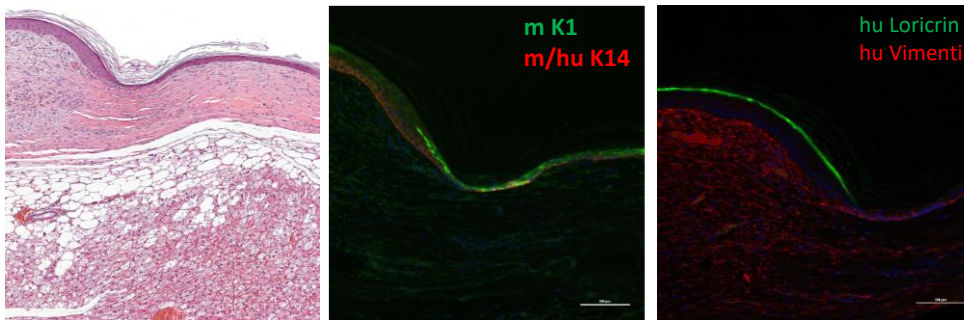


Fig. 15: Successful engraftment of genetically corrected organoid-derived RDEB fibroblast onto an immunocompromised mouse. Normal neonatal keratinocytes and corrected organoid-derived RDEB fibroblasts were grafted in a fibrin-based grafting system (see the previous report for details and Fig. 18A below). Six weeks post-grafting, the grafts were analyzed by H&E (left) and immunostaining. Middle: mouse-specific anti-K1 antibody (m K1, green) was used to mark human epidermis since this antibody only reacts with the mouse skin, and mouse/human-specific K14 (red) marks the epidermis. Right: human – specific vimentin (red) marks human dermis derived from corrected RDEB fibroblasts and human-specific loricrin (green) marks human epidermis. Scale bar, 100 μm.

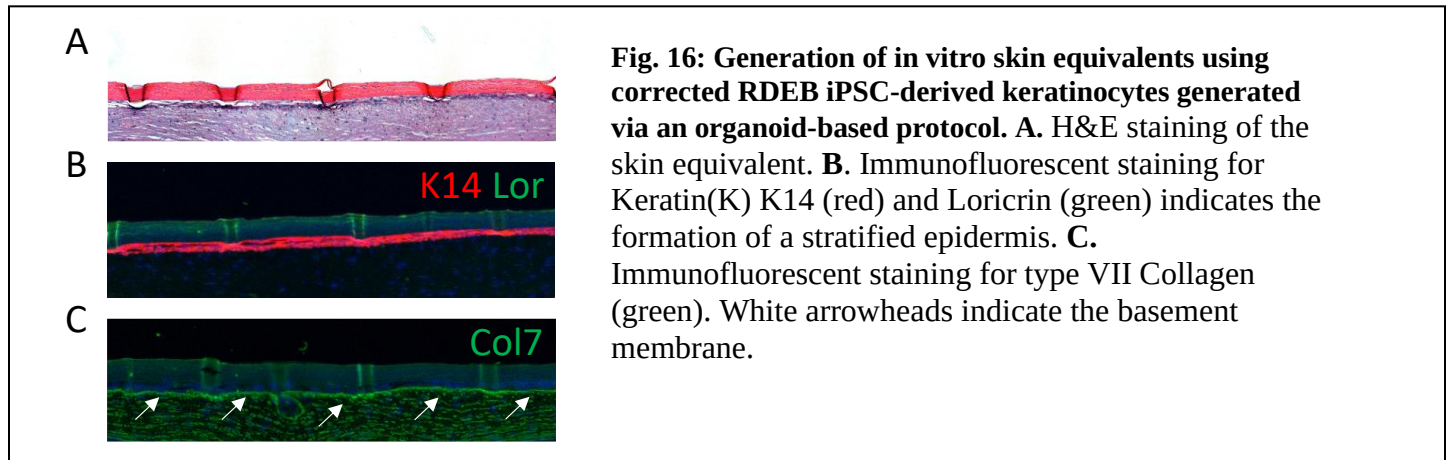
Task 3.3 To develop a cGMP-compatible protocol for the differentiation of COL7A1^{c.7485+G>A} corrected iPSCs into fibroblasts.

Since our differentiation protocol has changed, we are now focusing on the development of the enrichment strategy for fibroblasts derived from organoids. For that, we are utilizing the CD90 marker for MACS enrichment. This step is important to eliminate non-fibroblast cell types from the organoid culture. We found that our protocol for the isolation and culturing of organoid-derived fibroblast selects for dermal papillae (DP)

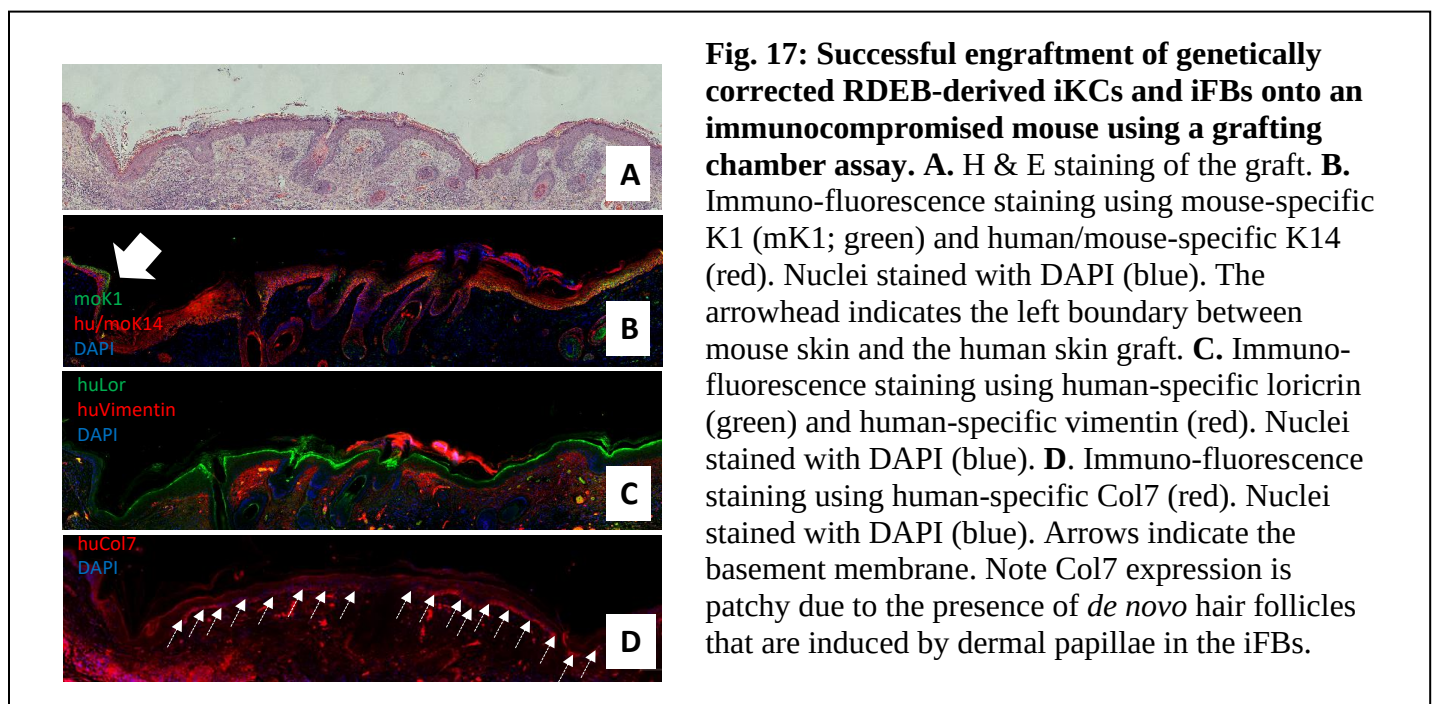
cells that can be isolated and used to produce de novo hair follicles and sebaceous glands (see previous report for the data). We will use these results in our new PRMRP expansion award, where we will develop a cGMP-compatible protocol for the isolation of DP cells..

Task 3.4 *To generate a composite graft using genetically corrected iPSC-derived keratinocytes and fibroblasts in organotypic cultures and verify type VII collagen deposition.*

Using organoid derived keratinocytes and fibroblasts we can now consistently generate composite grafts with a well-developed epidermis. We successfully generated in vitro 3D skin equivalents using cells derived from all our organoid differentiation experiments. Fig. 16 below shows a 3D skin equivalent generated from keratinocytes and fibroblasts derived from genetically corrected RDEB iPSC skin organoids. Therefore, this task has been accomplished.



Task 3.5 *To assess wound closure by iPSC-derived composite grafts in immunocompromised mice.* We successfully generated a composite graft from three genetically-corrected RDEB iPSC lines (**Fig. 17**). To date, all grafts formed with organoid-derived skin cells appear to be durable and persist on mice for up to 4 months. Therefore, this task has been accomplished.



Unfortunately, the time required to generate epidermal sheets and composite skin grafts from genetically corrected iPSCs is lengthy and consequently expensive due to the long period of use of a cGMP facility. The use of the FDA-approved “spray-on-skin” RECELL device would decrease the time to patient application vs. the time and cost it takes to grow epidermal sheets and would potentially produce superior outcomes for EB patients due to the lower risk of inflammation and scarring. In addition, composite skin grafts have a limited shelf-life and are difficult to transport, while the use of the “Spray-on- Skin” delivery approach would require just a cell suspension, which could potentially be frozen, stored and shipped around the country for administration onto patients. Using the funding from the NIH and support from AVITA Medical through a Sponsored Research Agreement, we established a novel mouse xenograft model suitable for testing the “Spray-On-Skin” Delivery approach of iKCs and iFBs and the generation of safety and efficacy data for the pre-IND meeting. The model is based on our previously published chamber-based xenografting assay that we modified by encapsulating each cellular component in corresponding fibrin gel/media mix and grafting each cellular component sequentially starting with fibroblasts. Using the funding provided by this award, we tested genetically corrected RDEB iKCs and iFBs in this fibrin-based assay and confirmed its applicability for the delivery of these cells. We have just published this model in BioProtocol. The manuscript acknowledges the current DOD award and the preprint is attached in Appendix 3 (**Pavlova, et al., The Development of an Advanced Model for Multilayer Human Skin Reconstruction In Vivo, BioProtocol, 2024**). This model will now be used for our new expansion award to test the safety and efficacy of organoid-derived keratinocytes, fibroblasts and dermal papillae cells.

Task 3.6 To assess tumorigenicity and the presence of residual iPSCs in the composite grafts.

An important safety concern of our therapy is the potential formation of benign teratomas due to contamination of undifferentiated iPSCs. We have made several key observations showing that the risk of contaminating iPSCs in the manufacturing process and their risk to form teratomas on the dermis is exceedingly low. First, after the CiniMACS prodigy enrichment of iKCs, the level of Tra1-60⁺ cells indicative of undifferentiated iPSCs is virtually undetectable (not shown). Second, we observe a substantial reduction of iPSCs grown in keratinocyte media. We have performed spike-in experiments of undifferentiated iPSCs with normal human keratinocytes in different ratios (0%, 0.5%, 5%, and 25%) in the media we are using for iKC expansion. At the end of 6-day culturing, flow cytometry analyses for the expression of the pluripotency markers SSEA3 and TRA-1-60 and the specific keratinocyte marker CD104 (integrin β4) was performed, and the fold reduction of iPSCs in mixed cultures was calculated as compared to the flow cytometry results obtained at the time keratinocytes and iPSCs were plated. We found that our culturing conditions promote ~900-fold reduction in the number of iPSCs (**Table 2**), suggesting that even if a few iPSC cells were left after differentiation, they would likely be eliminated by continuous culturing under keratinocyte growth conditions.

Table 2: 25% iPSC/75% Keratinocyte co-culture condition was used to calculate fold-reduction of iPSCs after 6 days. Fold-reduction was calculated for iPSCs defined as cells co-expressing SSEA3 and TRA-1-60			
	Day 0	Day 6	Fold Reduction of iPSCs
SSEA3+/TRA-1-60+	27.25%	0.03%	908

Finally, in our grafting experiment from Task 3.5, which was collected at 4 months post-grafting, we did not observe any tumor formation. To assess whether iPSC-derived cells migrate to other organs, which will be an indication of cell transformation, we established a human Alu-specific qPCR assay. Previous studies have verified the utility of this approach to detect a few human cells in rodent tissues (see Funakoshi et al. Scientific Reports 2017). Using primers and probe specific for human ALU repeats from the Funakoshi et al. study, the specificity and lower limit of detection of the assay was established using DNA from cultured human fibroblasts and mouse tissues from wildtype (wt) and skin cancer engrafted mice (see previous report for details). This assay will be used in our new award (PR222029) to assess the safety of organoid derived keratinocytes, fibroblasts and dermal papillae cells.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We anticipate that our project will develop a stem-cell based therapy for the treatment of epidermolysis bullosa (EB), a group of rare inherited skin blistering diseases. EB derives from genetic mutations in structural proteins of the skin and sentences those afflicted to a life of severe pain and disability due to constant blistering and scarring. The development of a stem-based therapy is a complex process that needs to be reproducible and performed under clinically relevant standards. During this award, we generated a panel of genetically corrected RDEB iPSCs using a cGMP-compatible protocol. We developed a cGMP-compliant protocol for the purification of iKCs using the CliniMACS Prodigy platform. We also developed a protocol for the isolation of functional iFBs and iKCs from organoids generated from genetically corrected RDEB iPSCs. In addition, we showed that the risk of contaminating iPSCs in the manufacturing process and their risk for forming teratomas is exceedingly low. The data generated with the current support and other sources allowed us to successfully compete for the FY2022 PRMRP Expansion Award (PR222029) and receive the funding to continue our efforts toward the clinical translation of our iPSC-based therapy for EB. Thus, the accomplishments achieved during this funding period will bring us a step closer toward approval for a clinical trial to treat EB.

What was the impact on other disciplines?

Stem cell-based strategies similar to the one proposed in this application, whereby patient cells are genetically corrected and reprogrammed into immature induced pluripotent stem cells (iPSCs) that can be subsequently differentiated into target cell types for transplantation, can be applied to virtually any other currently incurable monogenic disease, including cystic fibrosis, Fanconi anemia, beta thalassemia, etc. However, unlike other monogenic diseases, EB, and especially RDEB, may represent an ideal platform to initially test an iPSC-based therapy due to the orphan nature of EB and its severity. Our project provides important safety data that will be applicable for the use of iPSC-derived cells to regenerate other tissues and organs.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Due to inconsistent engraftment of iPSC-derived keratinocytes and fibroblasts generated using a conventional monolayer protocol, we re-evaluated our approach and focused on the optimizations of an organoid-based protocol for the generation of iPSC-derived keratinocytes and fibroblasts. These changes were technical in nature and did not affect the overall scope or specific aims of the application. These changes were listed during previous progress reports.

Actual or anticipated problems or delays and actions or plans to resolve them

Due to the COVID-19 pandemic, the shutdown of research laboratories, and the subsequent restriction on the number of research staff that could work at any given time, we faced a delay in accomplishing the goals of this project. To resolve this delay, we requested two no-cost extensions, which were both granted. These no-cost extensions allowed us to successfully complete the project without requesting any additional funding.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Neumayer G, Torkelson JL, Li S, McCarthy K, Zhen HH, Vangipuram M, Jackow J, Rami A, Hansen C, Guo Z, Gaddam S, Pappalardo A, Li L, Cramer A, Roy KR, Nguyen TM, Tanabe K, McGrath PS, Bruckner A, Bilousova G, Roop DR, Bailey I, Tang JY, Christiano A, Steinmetz LM, Wernig M, Oro AE (2024). A scalable, GMP-compatible, autologous organotypic cell therapy for Dystrophic Epidermolysis Bullosa. **Under Review** (Nature Medicine). (DOD support acknowledged).
2. Warshauer EM, Brown A, Fuentes I, Shortt J, Gignoux C, Montinaro F, Metspalu M, Youssefian L, Vahidnezhad H, Jacków J, Christiano AM, Uitto J, Fajardo-Ramírez ÓR, Salas-Alanis JC, McGrath JA, Consuegra L, Rivera C, Maier PA, Runfeldt G, Behar DM, Skorecki K, Sprecher E, Palisson F, Norris DA, Bruckner AL, Kogut I, Bilousova G, Roop DR (2021). Ancestral patterns of recessive dystrophic

epidermolysis bullosa mutations in Hispanic populations suggest sephardic ancestry. *Am J Med Genet A*. 2021 Aug 26;. doi: 10.1002/ajmg.a.62456. [Epub ahead of print] PubMed PMID: 34435747. (DOD support acknowledged).

3. Pavlova M, Balaiya V, Flores JC, Ferreyros M, Bush K, Hopkin A, Kogut I, Roop DR, Bilousova G (2024). The Development of an Advanced Model for Multilayer Human Skin Reconstruction In Vivo. *BioProtocol*, 2024, Jan 20. **In Press** (DOD support acknowledged).
4. Emily Mira Warshauer, Paul A. Maier, Goran Runfeldt, Ignacia Fuentes, María J. Escámez, Laura Valinotto, Mónica Natale, Graciela Manzur, Nuria Illera, Marta García, Marcela del Río, Ángeles Mencia, Almudena Holguín, Fernando Larcher, Garrett Hellenthal, Adam Brown, Liliana Consuegra, Carolina Rivera, Inês Nogueiro, Jean Tang, Anthony Oro, Peter Marinkovich, Francis Palisson, Matthias Titeux, Alain Hovnanian, Eli Sprecher, Karl Skorecki, David Norris, Anna Bruckner, Igor Kogut, Ganna Bilousova, Dennis R. Roop (2024). Secret Sephardic Origins Revealed for Rare Skin Disorder, Recessive Dystrophic Epidermolysis Bullosa, in Individuals Carrying the Unique c.6527insC Mutation. **Under Review** (DOD support acknowledged).
- 5.

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

1. Kogut I., McGrath P.S., Pavlova M., Butterfield K., Diette N., Bilousova G. and Roop D.R. (2018). Combining the reprogramming of RDEB fibroblasts with mono- and bi-allelic correction of the mutant COL7A1 gene into a one-step procedure. The 2018 Annual Meeting of the Society of Investigative Dermatology. *J Invest Dermatol* 138 (5), S72.
2. Torkelson J.L., Hansen C., Jackow J., Guo Z., Hui-Zhen H., Hayashi R., Sallee B., McCarthy K., Neumayer G., McGrath P.S., Bilousova G., Kogut I., Roop D., Christiano A., Wernig M and Oro A. (2019). Scalable production of CRISPR-corrected autologous iPSC derived skin grafts to treat epidermolysis bullosa. The 2019 Annual Meeting of the Society of Investigative Dermatology, Chicago, IL. *J Invest Dermatol*, 139 (5), S179.
3. McGrath P.S., Butterfield K., Pavlova M., Murthy M, Roop D.R., Bilousova G. and Kogut I. (2020). Combined RNA-based gene correction and reprogramming of RDEB fibroblasts into iPSCs for various COL7A1 mutations. The 2020 Annual Meeting of the Society of Investigative Dermatology, Virtual meeting. *J Invest Dermatol*, 140 (7), S1-S144.
4. McGrath P.S., Pavlova M., Kizub I., Ferreyros M., Butterfield K., Murthy M., Bruckner A.L., Kogut I., Bilousova G., and Roop D.R. (2020). State of the Art iPSCs and Alternative Methods for Delivering Cells Derived from Gene-Edited iPSCs. The EB 2020-6th World Conference of EB Research and '5th Conference of EB-CLINET, DEBRA International, Virtual Meeting, Moscow, Russia.

5. Pavlova M., Flores J.C., Vieau S, Balaiya V, McGrath P.S., Bush K., Hopkin A., Bruckner A.L., Kogut I., Roop D.R., and Bilousova G. (2022). A novel preclinical model for translating an induced pluripotent stem cell therapy for the treatment of skin diseases. The 2022 Annual Meeting of the Society of Investigative Dermatology, Portland, OR. *J Invest Dermatol*.
6. Pavlova M., Vieau S, Flores J.C., McGarvey S., Balaiya V, Murthy M., Schipper J., McGrath P.S., Bush K., Hopkin A., Bruckner A.L., Kogut I., Roop D.R., and Bilousova G. (2022). Developing new stem cell differentiation and validation platforms for translating an induced pluripotent stem cell therapy for the treatment of skin diseases. The 2022 ISSCR Annual Meeting. San Francisco, CA.
7. Maryna Pavlova, Jocelyn C. Flores, Velmurugan Balaiya , P. Sean McGrath, Kile Carson Butterfield, Shennea McGarvey, Sean Vieau, Anna Bruckner, Igor Kogut, Dennis Roop, Ganna Bilousova. Utilizing an organoid-based approach for the derivation of genetically corrected keratinocytes and fibroblasts in an induced pluripotent stem cell therapy for skin diseases. The 2023 Annual Meeting of the Society of Investigative Dermatology. Tokyo, Japan

- **Website(s) or other Internet site(s)**

<i>Nothing to Report</i>

- **Technologies or techniques**

We developed an organoid-based approach for the derivation of functional keratinocytes and fibroblasts from iPSCs. This approach will be shared with the research community through a publication, which is currently in preparation.

- **Inventions, patent applications, and/or licenses**

<i>Nothing to Report</i>

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Dennis Roop
Project Role: PI
Nearest person month worked: 0.3
Contribution to Project: Dr. Roop oversees the project as a PI.
Funding Support: National Institute of Health, EB Charities; institutional support, DEBRA International

Name: Ganna Bilousova
Project Role: Co-Investigator
Nearest person month worked: 3
Contribution to Project: Dr. Bilousova prepares regulatory compliance documents and oversees the work related to iPSC generation and differentiation.
Funding Support: National Institute of Health, EB Charities, institutional support, DEBRA International, DOD,

Name: Igor Kogut
Project Role: Co-Investigator
Nearest person month worked: 3
Contribution to Project: Dr. Kogut oversees a transfer of our technologies into a cGMP compliant facility.
Funding Support: National Institute of Health, EB Charities, institutional support, DEBRA International

Name: Maryna Pavlova
Project Role: RA
Nearest person month worked: 6
Contribution to Project: Dr. Pavlova works on the generation of skin organoid, differentiation of iPSCs into keratinocytes and xenografting. She also assists Dr. Bilousova in the generation of 3D skin equivalents.
Funding Support: National Institute of Health, EB Charities, DEBRA International

Name: Jocelyn Castillo Flores
Project Role: PRA
Nearest person month worked: 7.5
Contribution to Project: Ms. Castillo Flores assist Dr. Pavlova in iPSC-differentiation.
Funding Support: National Institute of Health, EB Charities

Name: Enrique Torchia
Project Role: Assistant Professor-Research
Nearest person month worked: 6
Contribution to Project: Dr. Torchia develops in vivo tumorigenicity assays to determine the safety of iPSC-derived cells.
Funding Support: DOD, institutional support

Name: Kiel Carson Butterfield
Project Role: Professional Research Assistant (PRA)
Nearest person month worked: 2
Contribution to Project: Ms. Butterfield assists Dr. Kogut in adapting our technologies to cGMP manufacturing.
Funding Support: National Institute of Health, CIRM, EB Charities

Name: Michael Ferreyros
Project Role: Sr. PRA
Nearest person month worked: 2
Contribution to Project: Mr. Ferreyros assists Dr. Bilousova in preparing regulatory compliance documents and maintaining cell lines. He is also involved in antibody testing for the project.
Funding Support: National Institute of Health, EB Charities

Name: Patrick S. McGrath
Project Role: Sr. Research Associate
Nearest person month worked: 3.25
Contribution to Project: Dr. McGrath is involved in designing and testing gene editing constructs for the project. He also assists Dr. Bilousova in molecular biology techniques and participates in a cGMP transfer of our technologies.
Funding Support: National Institute of Health, CIRM, EB Charities

Name: Shennea McGarvey
Project Role: PRA
Nearest person month worked: 2.0
Contribution to Project: Mrs. McGarvey assists Dr. Bilousova in differentiating iPSCs into fibroblasts and keratinocytes. .
Funding Support: National Institute of Health, EB Charities

Name: Josiah Fernandes
Project Role: PRA
Nearest person month worked: 0.8
Contribution to Project: Mr. Fernandez assists Dr. Bilousova in the generation of 3D skin equivalents .
Funding Support: National Institute of Health, EB Charities

Name: Chann Makara Han
Project Role: PRA
Nearest person month worked: 1.4
Contribution to Project: Mr. Han assists Dr. McGrath in analyzing off-target events in our Cas9-mediated gene correction strategy .
Funding Support: National Institute of Health, EB Charities

Name: Christopher Taylor
Project Role: PRA
Nearest person month worked: 2
Contribution to Project: Mr. Taylor assists Dr. Bilousova in mouse grafting experiments and maintenance of a mouse colony.

<i>Funding Support:</i>	<i>National Institute of Health, EB Charities, institutional support</i>
<i>Name:</i>	<i>Andrii Rozhok</i>
<i>Project Role:</i>	<i>Instructor</i>
<i>Nearest person month worked:</i>	<i>3.18</i>
<i>Contribution to Project:</i>	<i>Mr. Rozhok develops a platform to analyze whole-genome sequencing data.</i>
<i>Funding Support:</i>	<i>Institutional support</i>
<i>Name:</i>	<i>Velmurugan Balaiya</i>
<i>Project Role:</i>	<i>RA</i>
<i>Nearest person month worked:</i>	<i>1.6</i>
<i>Contribution to Project:</i>	<i>Dr. Balaiya performs in vivo xenografting experiments.</i>
<i>Funding Support:</i>	<i>Institutional support, Avita Medical, DOD</i>
<i>Name:</i>	<i>Sean Vieau</i>
<i>Project Role:</i>	<i>PRA</i>
<i>Nearest person month worked:</i>	<i>6</i>
<i>Contribution to Project:</i>	<i>Mr. Vieau assists Drs. Pavlova and Balaiya in in vivo experiments.</i>
<i>Funding Support:</i>	<i>DEBRA International, DOD</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

To continue the development of our iPSC-based therapy for EB, Dr. Roop has been awarded a PRMRP Expansion Award. Drs. Kogut and Bilousova are Co-Investigators. There is no research overlap with the current award since the new PRMRP application is a continuation of the current work.

PI: Dennis Roop – PR222029 (\$4,664,996)

a) Title: A Stem Cell-based Therapy for Recessive Dystrophic Epidermolysis Bullosa Delivered with a Spray on Skin Device

a) Agency: Department of Defense; FY 2022 PRMRP Expansion Award (Fort Detrick, MD 21702-5024)

b) Goals: The main goal of this application is to build upon our prior accomplishments and to adapt the production of organoid-derived genetically corrected RDEB iPSC keratinocytes and fibroblasts to cGMP-compliant manufacturing in the Gates Biomanufacturing Facility. **No overlap.** A new organoid-based approach will be used for the production of iPSC-derived keratinocytes and fibroblasts.

c) Aims: (1) To develop a cGMP-compatible protocol for the generation of hair-bearing skin organoids from genetically corrected RDEB iPSCs and for the isolation of keratinocytes and fibroblasts from these organoids. (2) To perform a small-scale cGMP-compatible run for the production of organoid-derived skin cells from genetically corrected RDEB iPSCs. (3) To develop a protocol for the isolation of dermal papillae cells from genetically corrected RDEB organoids. (4) To generate pre-IND safety and efficacy data.

d) Start and end date: 09/01/2023-08/31/2027

Dr. Roop has also received an award from the NIH to support the filing of an IND application and the initiation of the actual clinical trial. This award will not be used for the generation of IND-enabling data. Therefore, there is no scientific overlap with the current award or PR222029.

PI: Dennis Roop and Anna Bruckner (\$5,364,750)

a) Title: Study of the Safety, Tolerability, and Efficacy of an iPS Cell-based Therapy for Recessive Dystrophic Epidermolysis Bullosa Delivered with a Spray on Skin Device

b) Agency: NIH/NIAMS (Bethesda, MD 20892-3675)

c) Goals: The main goal is to perform a clinical trial to assess the safety, tolerability and efficacy of delivering genetically corrected iPSC-derived keratinocytes and fibroblasts to Recessive Dystrophic

Epidermolysis Bullosa patients with AVITA Medical's "Spray-on-Skin" device as a more straightforward and cost effective alternative to skin grafts. **No overlap.** The award will fund the preparation of documents for IND approval and a future clinical trial but not the generation and characterization of the therapeutic product.

d) Aims (1) Complete the preparations necessary to initiate the clinical trial (UG3 Milestones).

(2) Evaluate the safety of the treatment. (3) Evaluate the treatment site for graft survival and production of functional Col 7. (4) Evaluate preliminary clinical efficacy of the treatment by assessing wound closure and durability of healing.

e) Start and end date: 09/01/2023-08/31/2028

f) Level of effort: 8.33 %

g) Role: Co-PI

h) Overlap: None

What other organizations were involved as partners?

The project was initiated in collaborations with Stanford and Columbia Universities as subcontracts. However, due to the delay in getting ACURO approval, Dr. Oro (Stanford) and Dr. Christiano (Columbia) were not able to perform animal studies, only *in vitro* studies in Year 1. Due to the further delays in getting ACURO approvals for work to be performed at both Stanford and Columbia Universities, and onset of the COVID-19 pandemic in Year 2, Dr. Oro and Dr. Christiano were not able to perform the work agreed upon in their subcontracts. Therefore, with the approval of our PRMRP program officer and the DOD, we terminated the subcontracts with Stanford and Columbia Universities for Years 2 and 3, as well as for our no-cost extension requests.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Included with the report.

9. APPENDICES:

Appendix 1: Manuscript- Neumayer et al, 2024

Appendix 2: Manuscript- Warshauer et al., 2021

Appendix 3: Manuscript – Pavlova et al., 2024

Appendix 4: Manuscript- Warshauer et al., 2024

APPENDIX 1

Manuscript: Neumayer et al, 2024

A scalable, GMP-compatible, autologous organotypic cell therapy for Dystrophic Epidermolysis Bullosa

Gernot Neumayer^{*3}, Jessica L. Torkelson^{*1,2}, Shengdi Li^{*6}, Kelly McCarthy^{1,2}, Hanson H. Zhen^{1,2}, Madhuri Vangipuram³, Joanna Jackow^{5,9}, Avina Rami⁵, Corey Hansen⁵, Zongyou Guo⁵, Sadhana Gaddam¹, Alberto Pappalardo⁵, Lingjie Li¹, Amber Cramer¹, Kevin R. Roy⁴, Thuylinh Michelle Nguyen⁴, Koji Tanabe⁸, Patrick S. McGrath⁷, Anna Bruckner⁷, Ganna Bilousova⁷, Dennis Roop⁷, Irene Bailey^{1,2}, Jean Y. Tang^{1,2}, Angela Christiano⁵, Lars M. Steinmetz^{4,6}, Marius Wernig^{#3}, and Anthony E. Oro^{#1,2}

¹ Program in Epithelial Biology and Department of Dermatology,

² Center for Definitive and Curative Medicine,

³Institute for Stem Cell Biology and Regenerative Medicine, Department of Pathology, and
Department of Chemical and Systems Biology

⁴Department of Genetics and Stanford Genome Technology Center,
Stanford University School of Medicine, Stanford, CA 94305, USA.

⁵Department of Dermatology, Columbia University, New York, NY 10032.

⁶European Molecular Biology Laboratory (EMBL), Genome Biology Unit, 69117 Heidelberg,
Germany.

⁷Department of Dermatology, University of Colorado School of Medicine, Anschutz Medical
Campus, Aurora, Colorado, USA.

⁸I Peace Inc. Palo Alto, CA, USA

⁹St John's Institute of Dermatology, King's College London, London, UK

* # These authors made equal and independent contributions.

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Summary

Background. Gene editing in induced pluripotent stem (iPS) cells has been hailed to enable new cell therapies for various monogenetic diseases including dystrophic epidermolysis bullosa (DEB). However, manufacturing, efficacy and safety roadblocks have limited the development of genetically corrected, autologous iPS cell-based therapies.

Methods. We developed Dystrophic Epidermolysis Bullosa Cell Therapy (DEBCT), a new generation GMP-compatible (cGMP), reproducible, and scalable platform to produce autologous clinical-grade iPS cell-derived organotypic induced skin composite (iSC) grafts to treat incurable wounds of patients lacking type VII collagen (C7). DEBCT uses a combined high-efficiency reprogramming and CRISPR-based genetic correction single step to generate genome scar-free, *COL7A1* corrected clonal iPS cells from primary patient fibroblasts. Validated iPS cells are converted into epidermal, dermal and melanocyte progenitors with a novel 2D organoid differentiation protocol, followed by CD49f enrichment and expansion to minimize maturation heterogeneity. iSC product characterization by single cell transcriptomics was followed by mouse xenografting for disease correcting activity at 1 month and toxicology analysis at 1-6 months. Culture-acquired mutations, potential CRISPR-off targets, and cancer-driver variants were evaluated by targeted and whole genome sequencing.

Findings. iPS cell-derived iSC grafts were reproducibly generated from four recessive DEB patients with different pathogenic mutations. Organotypic iSC grafts onto immune-compromised mice developed into stable stratified skin with functional C7 restoration. Single cell transcriptomic characterization of iSCs revealed prominent holoclone stem cell signatures in keratinocytes and the recently described Gibbin-dependent signature in dermal fibroblasts. The latter correlated with enhanced graftability. Multiple orthogonal sequencing and subsequent computational approaches identified random and non-oncogenic mutations introduced by the manufacturing process. Toxicology revealed no detectable tumors after 3-6 months in DEBCT-treated mice.

Interpretation. DEBCT successfully overcomes previous roadblocks and represents a robust, scalable, and safe cGMP manufacturing platform for production of a CRISPR-corrected autologous organotypic skin graft to heal DEB patient wounds.

KEYWORDS

Induced Pluripotent Stem Cells (iPS cells), gene editing, CRISPR-CAS9, good manufacturing practices compatibility (c-GMP), tissue engineering, epithelium, skin, keratinocyte, dermal fibroblasts, wound healing, Epidermolysis Bullosa.

INTRODUCTION

Over the past decade, advances in the field of stem cell biology and regenerative medicine have enabled the prospect of genetically corrected autologous tissue replacement for previously untreatable conditions. Past clinical successes have mainly used viral gene transfer into somatic tissue, such as the bone marrow stem cells, illustrating that genetic correction of stem cells capable of tissue regeneration provides long-term disease modifying activity ^{1,2}. Somatic cell reprogramming into induced pluripotent stem (iPS) cells allows the generation of patient-derived, and thus autologous, iPS cells that can be genetically manipulated. iPS cells can be differentiated into not only individual cell types, but also organotypic cultures or organoids containing multiple key cell types that compose a tissue. However, such complex, multi-lineage manufacturing methods have not yet been developed at scale for clinical evaluation ³⁻⁵. The iPS cell-based approach provides a solution for the two main limitations of current somatic cell and gene therapy strategies: (i) iPS cells can be grown to virtually unlimited numbers providing a solid foundation for tissue organoid production-scalability and (ii) defined gene editing recreates a wild type allele while avoiding retroviral insertional mutagenesis. Thus, the combination of cell reprogramming, genomic correction of pathogenic mutations and composite cell transplantation has the potential to eradicate the impacts of disease-causing mutations in afflicted tissues ⁵⁻⁷.

While appealing in early studies, translation of corrected autologous iPS cell-derived products from proof-of-concept towards realistic clinical manufacturing has been met with a panoply of technical and regulatory roadblocks. Hurdles include developing a robust and reproducible manufacturing method that overcomes critical bottlenecks, including iPS cell generation, validated genetic correction, and safe and effective differentiation into desired tissues. A second set of hurdles includes sequential cell manipulation that results in protracted and labor-intensive manufacturing, increasing batch variability and compromising genomic integrity ⁸. Moreover, open questions regarding regulatory concerns, including determination of the safety risk of a pluripotent cell-derived product, avoidance of animal-derived products, and a risk assessment of genetic mutations introduced during cell culture and genetic engineering, have hampered widespread adoption of this cell/tissue therapeutic platform ^{9,10}.

The blistering disorder Dystrophic Epidermolysis Bullosa (DEB) maps to mutations in the *COL7A1* gene and results in extreme skin fragility due to collagen VII loss at the basement membrane zone (BMZ) ¹¹⁻¹³. Without curative treatment, the only option remains palliative wound care. Painful chronic wounds severely impact quality of life and the chronic inflammatory

milieu of constantly re- and degenerating skin wounds invariably results in the formation of an aggressive form of squamous cell carcinoma to which most patients ultimately succumb¹⁴⁻¹⁶. Disease severity and the lack of treatment options motivates the development of scalable and safe manufacturing options for autologous tissue replacement technologies¹⁷⁻¹⁹. Increasing efforts towards cell and gene therapies to treat Junctional EB have been led to great promise with the basal layer of the skin being self-renewing and demonstration that correcting the basal keratinocytes that contain holoclones with long-term stem cell activity has remarkable disease-modifying potential^{17,20,21}. In addition, a recent Phase I/IIA trial demonstrated that autologous grafts of expanded somatic RDEB keratinocytes transduced with a retroviral delivered *COL7A1* cDNA has highly efficient wound healing capability²². While these groundbreaking clinical trials showed disease-modifying activity, the approaches have important limitations including difficulty to reliably expand somatic RDEB keratinocytes and the safety concern of insertional mutagenesis by retroviral gene transfer^{23,24}. Development of clinically scalable iPS cell-derived skin replacement that overcomes current manufacturing challenges would represent a major advance for many genetic diseases, including DEB.

Here, we realize the advantages of an iPS cell-based multi-lineage differentiation approach to generate organotypic composite grafts via next generation genetic and cellular engineering. Solving critical bottlenecks, we refine a practical and simplified Good Manufacturing Practice (GMP)-compatible protocol for the generation of genetically corrected autologous organotypic skin grafts that include keratinocytes, dermal fibroblasts, and melanocytes for the long-term healing of DEB patient wounds.

RESULTS

Optimization of CRISPR/CAS9-mediated targeting of the *COL7A1* locus

While previous work^{8,25,26} demonstrated the possibilities of *ex vivo* autologous iPS cell-based gene therapy for treatment of Recessive Dystrophic Epidermolysis Bullosa (RDEB), several hurdles preventing clinical translation remained. These include: 1) relatively inefficient iPS cell derivation and genetic correction, and 2) lack of defined and efficient protocols for differentiation of edited iPS cells into multi-lineage induced skin composites (iSCs). Consequently, the previous protocols took many months to complete and involved multiple clonal steps, greatly increasing complexity and procedural variabilities, thereby complicating the development of Standard Operating Procedures (SOPs) while pronouncing the rate of culture-induced mutations⁸. To overcome these limitations, we evolved a next-generation, scalable, non-integrating, xeno-

free, and GMP-compatible platform that produces an epidermal-dermal-melanocyte containing, organotypic iSC product for long-term patient wound healing.

We first designed a cGMP method that allows derivation of *COL7A1*-corrected iPS cells from primary patient fibroblasts in 4 weeks. This SOP integrates iPS cell reprogramming and gene correction into a single manufacturing step (Fig. 1A), reducing culture time, mutational burden and clonal bottlenecks. In this design, primary patient fibroblasts from a dermal punch biopsy are transiently transfected with (i) CAS9-sgRNA containing ribonucleoproteins (RNPs), (ii) single stranded oligodeoxynucleotides (ssODNs) that encode the desired genomic correction, and (iii) reprogramming factors-encoding mRNAs that induce iPS cells. iPS cell colonies, emerging ~14 days after the initial transfection with reprogramming factors, are then isolated and screened via droplet digital (dd) polymerase chain reaction (PCR) employing probes specific for the properly corrected *COL7A1* locus. Ensuing quality controls validate *COL7A1* edits, cellular identity, and genomic/chromosomal stability.

To test the applicability of this approach, we assessed the potential for gene targeting of the *COL7A1* locus in patient-derived dermal fibroblasts from three individuals carrying the so-called “Colorado” mutation, i.e. *COL7A1* c.7485+5G>A (Fig. 1B). Patients CO1 and CO2 carry homozygous and patient DEB125 carries a compound heterozygous “Colorado” mutation (*COL7A1* c.6527dupC is the other pathogenicity of DEB125). Initially we tested all possible sgRNAs mediating CAS9-cutting of the Colorado-allele and ssODNs of various lengths encoding for either the (+) or (-) strand of DNA (Fig. 1B). While the 6 possible sgRNAs specific to the Colorado mutation exhibited favorable *in silico* predicted specificity and activity scores (Fig. S1A), Tracking of Indels by Decomposition (TIDE) analysis in patient fibroblasts transfected with RNPs showed variable efficiencies (Figs. 1C, S1B-C). Focusing on the two most efficient sgRNAs (i.e. sgRNA C2 and C4), we analyzed their specificity for the Colorado allele (Fig. 1D). TIDE analysis of wild type (wt), heterozygous DEB125, and homozygous CO2 fibroblasts transfected with RNPs revealed that sgRNA C4, with a protospacer adjacent motif (PAM) closer to the Colorado mutation, is more specific for the disease allele.

Next, we optimized sgRNA C2 and C4 RNP-mediated repair of mutant *COL7A1* using ssODNs encoding for the wt *COL7A1* sequence and 4 silent mutations used for detection of editing events via specific ddPCR probes (Fig. 1B). By comparing with a bi-allelic reference locus, ddPCR allowed quantification of the edited *COL7A1* allele. This approach showed that sgRNA

C2 mediated 2-2.5x more *COL7A1* targeting events than sgRNA C4 (Fig. 1E). The ssODN length (up to 200 nt) positively correlated with editing efficiencies (Fig. S5B-C). Surprisingly, also the strandedness of ssODNs influenced editing efficiencies with the (+) strand-encoding sequence yielding 2-2.5x higher efficiencies than the (-) ssODN (Fig. 1E) in both homozygous and heterozygous patients (Fig. S1D). We confirmed the ddPCR results by cloning the target locus of bulk-edited fibroblasts into plasmids, followed by analysis of individual, cloned alleles via PCR primers specific for the edited locus (Figs. 1F, S1F-H). Analysis of 77 individual target alleles each, from cells treated with all possible combinations of sgRNA C2 or C4 and ssODN (+) or (-) validated the ddPCR results. Sanger sequencing revealed that both, sgRNA C2 and sgRNA C4 are mediating *COL7A1* editing as intended when used in combination with (+) ssODNs (Figs. 1G-H, S1F-H). Remarkably, although all the Sanger-sequenced alleles from cells treated with (-) ssODNs exhibited at least partial integration of donor sequences, none of them was correctly edited (not shown). We conclude that all parameters involved, i.e. the sgRNA sequence, the length and strandedness of ssODNs, and the particular target locus must be tested for optimal efficiency and specificity of editing events.

Combining iPS cell-reprogramming and *COL7A1* correction in one manufacturing step

The optimized *COL7A1* targeting efficiency allowed us to test whether it may be feasible to correct and reprogram cells in a single manufacturing step, which would greatly minimize production time and eliminate multiple clonal selections. We chose to deliver the reprogramming factors via transfection of mRNA since this approach has been shown to be efficient, is compatible with GMP manufacturing using chemically defined reagents, and represents a transient treatment leaving no genetic scars²⁶. In line with the TIDE assay in fibroblasts (Fig. 1D), sgRNA C2-mediated *COL7A1*-editing in DEB125 fibroblasts directly followed by mRNA reprogramming resulted in ~15% targeted iPS cell colonies but was not specific for repairing the mutant allele of this heterozygous patient (Fig. S2). We therefore focused on sgRNA C4, which mediates only slightly lower editing efficiency but is more specific for the Colorado allele. First, we determined via a dose range that 5 pmole sgRNA C4-containing RNP per 30k fibroblasts was sufficient for optimal cutting of the Colorado allele (Fig. S1E). Next, we measured the *COL7A1* editing efficiencies in fibroblasts of patients CO1 and CO2, which carry a homozygous Colorado mutation, using engineered high fidelity CAS9s. Reassuringly, ddPCR, using ssODN (+) and sgRNA C4 in complex with CAS9s HiFi or SpiFi showed targeting events in 3-7% of the cells (Fig. 2A-B). These encouraging efficiencies prompted us to test induction of reprogramming immediately following *COL7A1* editing via GMP-compatible SpiFi CAS9. We

screened 186, 293, and 24 iPS cell clones derived from patient CO1, CO2 and DEB125 by ddPCR, yielding 8, 25, and 1 candidate lines, respectively (Fig. 2C, G and S4). Analysis of 5, 11, and 1 candidate iPS cell lines from each patient via conventional PCR amplification of the target locus, followed by plasmid cloning and Sanger sequencing of individual alleles, revealed that all but 2 lines were correctly edited (Fig. 2D-G). As expected from the specificity and efficiency mediated by guide C4 (Fig. 1D), in iPS cell lines from patients homozygous for the Colorado mutation (i.e. CO1 and CO2) the second allele acquired InDel mutations of various sizes in all cases, whereas the second, wt allele in iPS cells derived from the heterozygous patient DEB125 remained unperturbed. Of important note, in some iPS cell clones derived from homozygous Colorado patients the InDels on the second allele were larger than the 731bp PCR amplicon that we initially chose for analysis (Fig. 2D), wrongly implying bi-allelic *COL7A1*-correction. Larger PCR amplicons identified some of these bigger InDels, e.g. a 654bp deletion in line CO2-65(B), while the nature of others (e.g. line CO1-48) could not be determined (Fig. 2E-G; see discussion).

Next, we chose candidate iPS cell lines for deeper genomic characterization. Sanger sequencing of up to 28 individual target loci cloned into plasmids showed an equal distribution between correctly edited and the second allele in all but one sample (i.e. CO2-89(A); Fig. 2G). In iPS cell lines derived from homozygous patients, the second allele that did not incorporate the ssODN always displayed a characteristic InDel, indicating high efficiency of employed RNPs. Other than iPS cell line CO2-89(A), in which 20% of sequences displayed deletions of a different nature (not shown), our data is consistent with clonal origin of picked iPS cell lines. In addition, we verified cellular identity of our iPS cell lines by robust expression of the pluripotency markers TRA-1-81, TRA-1-60, NANOG, *Lin28*, *Oct4*, and *Sox2* as determined by immunofluorescence, cytometry, and/or RT-PCR (Fig. 2H-I, S3D, S6A).

Finally, to test applicability to correct other mutations, we followed the same SOPs developed for the Colorado mutation and successfully derived one-step corrected iPS cell lines with similar efficiency from a fourth patient, DEB135, who carries two different compound heterozygous mutations, i.e. *COL7A1* c.6781C>T and c.6262 G>A (Fig. S5). Importantly, this demonstrates that our single clonal step iPS cell manufacturing process can in theory be adapted for other therapeutic cell manufacturing procedures.

Scalable and reproducible differentiation of DEB iPS cells into organotypic skin grafts

Proper mammalian skin development requires the interaction between early surface ectoderm progenitors, regional mesoderm, and neuroectoderm²⁷⁻³⁰. We recently demonstrated the importance of a subtype of mesoderm that depends upon the chromatin regulator Gibbin for proper epidermal stratification³¹. We therefore sought to develop a novel multi-lineage cutaneous organoid differentiation method that imitates the interaction and co-dependence of the cell lineages that cooperate in the embryo to form skin (Fig. 3A)^{31,32}. First, embryonic surface ectoderm was induced with retinoic acid/bone morphogenic protein-4 (RA/BMP4) for 7 days³¹. scRNA-seq analysis of day 7 cultures showed the successful creation of surface ectoderm, mesoderm, and neuroectoderm (Fig. 3B,C). The second inductive phase used a defined matrix and media containing the epidermal growth factors insulin, EGF and FGF for an additional 40-45 days, allowing reciprocal epithelial-mesenchymal-neuroectodermal interactions to mature the cultures into a therapeutic organotypic induced skin composite (iSC).

A common hurdle in pluripotent cell differentiation comes from stochastic mechanisms during complex cell culture that lead to variable keratinocyte maturation and presence of immature K14⁺, K18⁺ epithelial cells (Fig.3D)³³. To overcome this hurdle and to enrich for mature basal keratinocytes, we used the previously characterized stem cell surface marker ITGA6 /ITGB4^{34,35}. We verified that ITGA6 is expressed highly on p63⁺; K14⁺; K18⁻ cells, while K18⁺ cells were ITGA6 low or negative. An ITGA6 magnetic bead-based, automated pro-separator AutoMACS® efficiently enriched for p63⁺; K14⁺ cells and removed K18⁺ cells (Fig. 3E-G, and S6B-C). In contrast to unenriched populations, ITGA6-enriched cells produced robust stratified epidermis/dermis in liquid/air interface organotypic cultures as demonstrated by involucrin expression and deposition of collagen VII to the BMZ (Fig. 3G-H). Importantly, mesodermal and melanocytic cell populations were still present after ITGA6-enrichment allowing continued signaling between cell types (Fig. 3L,K). We successfully differentiated and AutoMACS®-enriched five independent, genetically corrected DEB patient cells lines in multiple replicates demonstrating reproducibility among different lines from different patients. The “coupling efficiency”, i.e. the ratio between ITGA6-enriched cells after differentiation and the input iPS cells, ranged between 40-130% (Fig. 3I, J), demonstrating the robustness of the process.

We next sought to implement ITGA6 enrichment at a clinical manufacturing scale. We performed 5 large scale differentiation runs that improved iSC formation by employing a CliniMACS® Plus cell separator in 3 different modes, yielding various enrichment and cell viability ratios. Limited cell expansion *in vitro* generated the needed cell numbers for future

clinical trials (Fig. S6D-H). Flow cytometry and bulk RNA-seq verified successful enrichment (Fig. S6B,E,H), demonstrating the feasibility of clinical scale manufacturing.

Lastly, we used single cell transcriptomics to characterize the final post-enrichment iSC, which would be equivalent to the DEBCT clinical product (Fig. 3K,L). We profiled iSC cultures from H9 cells and 5 patient iPS cell lines, revealing 8 distinct cell clusters representing surface ectodermal (C1, C2, C4, C7), mesodermal (C3, C5, C8) and melanocyte cell types (C6) (Fig. S6I) ^{31,36-40}. C1 most closely resembles basal keratinocytes (high K14, low K18, low cell cycle markers), C2 resembles long-term proliferative “holoclone” keratinocyte stem cells (high CDK1 and TOP2A; Fig. S6I) ^{41,42}. Cells in C4 and C7 initiated signatures of the early epidermal stratification phase. C5 and C8 cells closely resemble Thy1/CD90+ Gibbin-dependent mature dermal fibroblasts that are required for epidermal induction ³¹, and the C3 cluster contains cells of more immature dermal / pre-vascular characteristics ³⁶. Intriguingly, the 6 cell lines produced different ratios of these induced cell clusters, allowing us to correlate the functional implications of their presence (Fig. 5C-F).

Genomic stability of *COL7A1*-corrected iPS cells and iSCs

A critical safety aspect of cell expansion is genomic and chromosomal stability, as we estimate that 3×10^7 undifferentiated iPS cells are needed to generate one clinical iSC application of a 6x8 cm sheet graft in a Phase I/IIa trial (Fig. S6G ¹⁹). Unlike previously tested media ⁸, a more recently developed chemically defined media on plates coated with the E8 fragment of Laminin-511 ⁴³ allowed expansion of karyotypically normal iPS cell lines derived from 5 individuals with 3 sgRNAs to at least 3×10^7 cells in 11 of 12 instances (Figs. 4A, S7A). As part of our product safety methodology, we performed whole genome sequencing (WGS, 40x coverage) of four single-step corrected iPS cell lines from three patients carrying the Colorado mutation, their parental fibroblasts and differentiated ITGA6-enriched iSCs. WGS data confirmed all CAS9-mediated *COL7A1* edits (Fig. 2G). To identify novel single nucleotide variants (SNVs) and insertions or deletions (InDels) with high confidence, we used the agreement between three variant callers and filtered out all SNVs/InDels previously annotated in SNV/InDel databases (see material and methods). This strategy yielded an average of 70,386 $\pm$ 570 (SEM) novel SNVs and 32,465 $\pm$ 1,506 (SEM) novel InDels in each of the 11 samples. To find SNVs and InDels that are present specifically in either iPS cells and/or iSCs and thus, may be induced and/or selected for by our manufacturing process, we employed two alternative and complementary strategies: (i) k-means clustering by allele frequency (AF) and (ii) AF/odds ratio

cut-off filtering. The k-means clustering at high resolution (feature space $k=9$) showed that most SNVs/InDels exhibit similar AFs among all three cell types, suggesting that these variants are heterozygous or homozygous pre-existing germline variants (Fig. 4B and Fig. S7B). Only one cluster (cluster 7 for DEB125-1, Fig. 4B) contained iPS cell/iSC-specific variants and no cluster was identified with variants unique to either iPS cells or iSCs. The same pattern was observed in all four experiments. Importantly, there were only three iPS cell/iSC-specific variants found in more than one patient (Fig. S7C). Of these three variants, one shared between DEB125 and CO2 maps to an intron of *Magi2* and is already present in the respective parental fibroblasts at $AF=0.1-0.2$. Similarly, the two variants shared between lines CO2-65(B) and CO1-173, mapping to an intron of a ncRNA (i.e. LOC100507053) and an intergenic region, are also found in parental fibroblasts at $AF=0.2-0.3$, and $AF=0.1-0.2$, respectively. Thus, all three variants, themselves of unknown significance, were in fact already pre-existing in parental fibroblasts at lower AFs and are not introduced *de novo* by our manufacturing process.

Reassuringly, our second strategy based on AF/odds ratio cutoff-filtering identified >89% of the variants called by k-means clustering as shared iPS cell/iSC-specific (Fig. S7D). Unlike k-means clustering, AF/odds ratio cutoff-filtering identified variants unique to iPS cells or iSCs only, albeit in much smaller numbers than shared iPS cell/iSC-specific variants (Fig. 4C). Of note, almost all iPS cell-specific variants were present in other cell types with an $AF>0$, suggesting that these variants were not introduced *de novo* (Fig. S7E). In contrast, shared iPS cell/iSC- and iSC-specific variants were infrequent in other cell types suggesting some of them are *de-novo* or amplified from a rare ($AF<5\%$) pre-existing variant (Fig. S7E). There was no overlap of these variants between patients (Fig. 4C). Most cell type-specific variants map to intronic or intergenic regions. Gene ontology (G.O.) term analysis of all variants located at loci with predicted functional properties (i.e. exons, splice sites, UTRs, and promotor/enhancer elements) did not yield any significant enrichment (Fig. 4C).

To investigate potential guide-dependent CAS9 off-target mutations, we searched a window of 25 bases around all shared iPS cell/iSC variants for PAM-like NRG-motifs that are combined with sequence similarities to the used sgRNA C4⁴⁴. We did not observe any NRG motif adjacent to a 20-mer containing less than 6 mismatches compared to the sequence of sgRNA C4 (Fig. 4D). The cutting efficiency of CAS9 misguided by sgRNAs containing more than 3 mismatches is reported to be exceedingly low^{45,46}. Accordingly, TIDE analysis of fibroblasts transfected with sgRNA C4/CAS9 RNPs did not detect any significant InDel formation at any *in*

silico predicted exonic or intronic off target, whereas the *COL7A1* on target site was cut with over 87% efficiency (Fig. 4E and G). As TIDE can detect a maximum InDel size of 50bp, we also visualized InDels via plotting the normalized coverage of WGS reads obtained from unperturbed fibroblasts, thereof derived iPS cells and iSCs around all *in silico* predicted exonic, intronic, and intergenic off targets and the *COL7A1* on target. All previously identified CAS9-mediated deletions on the non-repaired *COL7A1* allele displayed the expected decreased read coverage (Fig. 4F, compare to Fig. 2G). No other InDels in proximity of potential off target cut sites were observed when searching a 1kb or 1Mb window (Fig. 4G and data not shown).

The functional consequences of detected variants are hard to predict. Their random nature and frequent pre-existence in the heterogeneous patient fibroblasts suggests that pathogenic effects caused by our manufacturing process are unlikely to materialize. To exclude, however, the *de novo* introduction or clonal expansion of variants in potentially cancer-promoting genes we performed the Clinical Laboratory Improvement Amendments (CLIA)-accredited, high-coverage sequencing Stanford Actionable Mutation Panel for Solid Tumors (STAMP)⁴⁷. iPS cell lines DEB125-1, DEB135-10(B), DEB135-24(B), CO1-131 and CO1-173 displayed no STAMP-hits other than germline variants already present in unperturbed parental fibroblasts (Figs. 4H, S5H). iPS cell lines CO2-65(B) and CO2-48(C) and their iSC progeny exhibited a heterozygous mutation in the androgen receptor (AR) that STAMP did not detect in fibroblasts (Fig. 4H). Targeted ddPCR analysis of this mutation in parental fibroblasts revealed however an AF of 3% of this variant (which is below the 5% detection limit of STAMP), suggesting clonal expansion of a rare somatic mutation or even a pre-cancerous lesion of this patient. Accordingly, many other iPS cell lines derived from patient CO2 by the same production run did not harbor this AR mt (Fig. S7F-G). These results highlight the merit of the STAMP oncopanel to exclude rare cell products with potentially pathogenic mutations.

***In vivo* efficacy and favorable safety profile of patient-derived *COL7A1*-corrected organotypic skin grafts**

To test the functionality of the corrected organoid iSCs, we transplanted ITGA6-enriched iSC cultures on the back of immunocompromised nude mice. With 3-12 manufacturing runs per line, all five *COL7A1*-corrected patient iPS cell lines produced viable grafts and formed human skin *in vivo* (Fig. 5A,B). Graft success was determined by formation of stratified epidermis consisting of K14⁺ basal cells and K10/Involucrin⁺ upper stratified layers, and detection of human-specific collagen 7 in the basement membrane zone (Fig. 5B). Successful grafts were stable for at least

1 month after transplantation and comparable to primary human keratinocyte grafts^{8,48,49}, thereby providing a biologically and clinically relevant endpoint.

While our manufacturing led to graftable iSCs from each line, we observed a line-dependent range of grafting efficacy (Fig. 5A). The different distribution of cell identity among the five lines characterized (Fig. 3K,L) enabled us to correlate the 4 epidermal, 3 dermal and 1 melanocyte cell groups of each line with its engraftment success rate. One prominent variable was the melanocyte cluster but that did not correlate with graftability (Fig. 5C, Fig. 3,KL). However, the lines with lowest grafting efficiency (CO1-173, CO1-131) distinctly lacked the two Gibbin-dependent fibroblast populations C5/C8, which were present in the other three lines (Fig. 3K,L, and 5C). Moreover, graftability correlated with the Gibbin-dependent dermal gene signature (Fig. 5D). To independently verify this finding, we measured the amount of Gibbin-dependent mesoderm in 4 iSC lines (n=3-7) by flow cytometry using the Gibbin-dependent marker CD90/Thy1 (Fig. S6I, Fig. 5E-F). Again, the two low efficiency lines exhibited few CD90+ cells whereas the better performing lines consistently produced 2-5% CD90+ dermal cells (Fig. 5E-F). These data suggest that optimal graftability requires the presence of Gibbin-dependent dermal cells, which have been shown to provide a critical maturation signal for epidermal stem cells³¹.

The main safety concern of the iSC product is tumor formation. To assess potential teratoma formation from residual undifferentiated iPS cells, we first evaluated pluripotency marker expression in expanded ITGA6-enriched cells. *Lin28A*, *Nanog*, *Oct4*, and *Sox2* were below detectability in differentiation experiments of five different cell lines (Fig. 5G). Utilizing RNA expression of the pluripotency marker *Lin28A* we measured the limit of detection of contaminating iPS cells at 0.38% (i.e. 1 iPS cell in 260 immortalized keratinocytes⁵⁰; Fig S8A). In combination with absence of ITGB4 in iPS cells, which is necessary for adhesion to the BMZ^{34,35}, we predicted the likelihood of teratoma formation to be low. Indeed, no teratomas were found in grafted animals. Another potential risk is the formation of squamous cell carcinoma (SCC), a common complication within the chronic wounds of adult EB patients. Careful investigation failed to show any histopathological signs of SCC formation in any of the human organoid skin grafts (Fig 5H). To assess the sensitivity to detect potential tumors in this assay, we performed spike-in positive control experiments using RDEB patient-derived SCC cells⁵¹. This defined the limit of detection (LOD) within the graft at 1 month at 0.025% SCC cells (Fig. S8B-C). To address the possibility of potentially metastasizing tumor cells from graft sites we designed a method to detect human DNA by Alu sequence qPCR from 7 organs and blood from

all grafted mice at 1, 3 and 6 months (Fig. 5H-J). Spike-in experiments using 1-10,000 iSCs in 500K mouse cells from various organs demonstrated the LOD as 3,000 iSCs for lymph node, brain, and liver. Sensitivity for quantitation was determined to be 10,000 iSCs at 38 PCR cycles for all four organs tested (Fig. S8D). Example Alu-qPCR result for the DEB125-1 iSC grafted mice at 1 and 6 months showed no detection for human Alu sequences from the mouse organs or blood, with all iSC grafts tested. The 4 patient lines, grafted on mice, were all assayed for Alu-qPCR with no detection (Figs. 5H-J, S8E). To the level of detectability our data reinforces the safety and efficacy of our cell manufacturing method.

DISCUSSION

With DEBCT manufacturing, we overcome existing technical hurdles to develop a scalable, GMP-compatible, and efficient platform for derivation of autologous and genetically corrected organotypic skin grafts for long-lasting treatment of RDEB patient wounds. This platform combines the generation and genetic correction of patient-derived iPS cells in a single manufacturing step and details a strategy for safe and reproducible manufacturing of graftable organotypic skin composites at clinical scale. The therapeutic product is composed of basal keratinocytes, dermal fibroblasts, and melanocytes, better resembling the composition of physiological tissues than previous approaches. Moreover, our use of generalizable manufacturing reagents and development of efficacy, toxicology and product characterization assays provide a reproducible regulatory and manufacturing path.

An important aspect of this study is the successful combination of CRISPR/CAS9-mediated gene editing and reprogramming into a single manufacturing step. Previous approaches generated iPS cells to be corrected in a subsequent clonal step, necessitating longer manufacturing time, higher cell expansion rates, and extensive additional quality control and release tests. Methodological simplification results in a series of advances: First, several new reagents enable clinical-scale corrected iPS cell manufacturing, including a novel transfection reagent that allows efficient delivery of three different nucleic acid/protein cargos with low toxicity, the high efficiency mRNA reprogramming kit, and the chemically defined iPS cell expansion media that ensures genomic/chromosomal stability. Second, the *COL7A1* correction and iPS cell reprogramming steps are seamless and lack genomic alterations to the DNA other than the corrected mutation and designed silent point mutations that facilitate genotyping. Hence, our approach removes potential complications associated with most other gene and cell therapy approaches caused by random insertion mutagenesis, introduction of non-physiological

gene regulatory elements, or alteration of endogenous loci. Third, genetically corrected autologous iPS cell banks can now rapidly be obtained and characterized in less than a month after dermal punch biopsy. This substantial acceleration not only increases the cells safety profile by lowering culture-induced mutation burden ⁸, but also reduces the time/cost of autologous iPS cell manufacturing, increasing the feasibility of this approach compared to off-the-shelf allogeneic iPS/ES cell-derived methods that suffer from host tissue tolerance issues ⁵².

We discovered one important caveat of CAS9-mediated editing. Whenever we observed integration of ssODN sequences on one allele, we almost always found InDel mutations on the other, which agrees with error-prone non homologous end-joining (NHEJ) being the predominant repair mechanism for DNA breaks in human cells ⁵³. These InDels can be very large (Fig.2), resulting in standard PCR genotyping failing to amplify the affected allele, giving the false impression that genetic correction was bi-allelic. Our quantitative ddPCR analysis indicated that of 479 iPS cell lines derived from homozygous patients, only 3 exhibited potential bi-allelic integration of donor sequences (i.e. <1%; Fig. S4). This contrasts with reports of significantly higher bi-allelic *COL7A1* editing events in primary RDEB keratinocytes and iPS cells. Studies using non-quantitative PCR genotyping approaches may want to consider this potential problem. We suggest to carefully characterize the other allele and target the downstream of the two compound heterozygous mutations to minimize potential undesired consequences of InDel mutations ⁵⁴⁻⁶⁰.

Our cell manufacturing method for deriving engraftable organotypic skin equivalents is tailored for scalable GMP production in a 45-day process that is defined, xenofree, extensively validated ^{61,62} and overcomes two key cell manufacturing hurdles. First, the induction of surface ectoderm, mesoderm and neuroectoderm mimics developmental signaling required for proper tissue maturation including development of epidermal, dermal, and melanocyte precursors. We find a significant proportion of the final iSC product to be the holoclone population previously identified as the long-term keratinocyte maintenance population ^{41,42}. In contrast to the previous culture intensive methods of subcloning and the use of a non-reproducible mouse fibroblast feeder line for maintenance, our inductive method produces all cell types necessary for an organotypic therapeutic product in scalable quantities via a defined and xenofree method. In addition, our studies support our previous results that underline the importance of epidermal-mesodermal signaling for subsequent induction of the region-dependent epidermal stratification program ³¹, as iPS cell clones deficient in differentiating into the Gibbin-dependent dermal population had a

lower grafting efficacy. The reason for this line-to-line variability remains undetermined but the identification of the Gibbin-dependent CD90 surface marker, expressed on a small subpopulation of necessary mesodermal-like iSC cells, may provide a quantifiable biomarker predicting efficacy of graftability. Future advancements may include identification of distinct mesodermal signals that could give rise to distinct epidermal stratification programs, facilitating more subtle tissue morphologies with minimal morphogen reagent concentrations.

Second, incorporation of our iSC stem cell enrichment step overcomes maturation heterogeneity that reduces epidermal polarity and graft stratification. Despite a defined multi-lineage organoid culture aimed to regulate epidermal-dermal induction, immature cellular products appear to dominantly reduce the polarity and stratification abilities, requiring their removal for optimal grafting performance. While the differentiation efficiency varied between patient clones, our development of an ITGA6 enrichment step allowed derivation of organotypic skin grafts at clinical scale from all patients included in the study. Cell sorting technology for enriching antigen-specific cells is being used in various approved clinical products including CAR T cell and CD34+ cell enrichment⁶³. Because ITGA6 and its binding partner ITGB4 are present at elevated levels on most epithelial stem cells, our enrichment method will be applicable to other epithelial tissues.

While our abbreviated manufacturing process greatly reduced the number of cell doublings and consequently the chance of culture-induced mutations⁶⁴, our detailed characterization by whole and targeted genome sequencing paired with animal toxicology provides a rigorous assessment of manufacturing mutational and associated pathological risk. A key question was whether genetic variants in the therapeutic product are introduced during cell manufacturing or were pre-existing in patient skin cells. Sensitive ddPCR and WGS techniques confirmed that many variants stem from clonal amplification of pre-existing somatic mutations rather than *de novo* manufacturing-induced mutations. In agreement with high genomic stability within our manufacturing process, we found no variants unique to iPS cells only. We note that in line with naturally occurring proliferation-induced random mutagenesis⁶⁴, a small fraction of all detected variants does, however, arise *bona fide*, as indicated by rare iSC-specific variants that do not have any reads in parental fibroblasts and clonal parental iPS cell lines (Figs. 4C, S7). We did not observe any overlap of such iSC-specific variants between lines derived from different individuals or the same patient. This reflects the random nature of naturally occurring somatic mutations and legitimates our advancement that now allows DEBCT manufacturing in an

unprecedented short time, thereby minimizing the risk of introducing a haphazard mutation with deleterious consequences. We did observe a three-fold line-to-line variability in the total number of SNVs arising in the different iPS cell lines (~1,300 to ~3,900). However, since many variants arise from clonal amplification of pre-existing somatic mutations, we conclude that the variation derives from the different mutational load of patient fibroblast.

Only three variants in only one detection method (k-means clustering) were found to be shared between patients and all of them were pre-existing. Similar results were found by the CLIA-certified STAMPv2 next generation sequencing assay to detect cancer-driving variants. Corresponding to our sequencing analysis, sensitive toxicology assays for residual pluripotent or carcinogenic cellular components found no evidence for local or distant invasive properties of the product. This included grafts derived from patient cells harboring a mutation in the androgen receptor, which scored positive on the STAMPv2 screen. Given the greater ease and clinic-ready nature, we conclude that the STAMPv2 variant detection assay, along with our toxicology assays, will ensure a well-defined cellular product with low risk originating from our iPS cell-derived tissue manufacturing methods.

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DECLARATION OF INTERESTS

The authors declare no competing or conflicting interests.

MATERIALS AND METHODS

Derivation and culture of primary patient fibroblasts: Fibroblasts from patients DEB125 and DEB135 were derived from a fresh dermal punch biopsy. Minced pieces (~1mm³) of biopsies were cultured in DMEM (Gibco 12-430-062) with 10% fetal bovine serum (FBS; Hyclone

SH30406.02 New Zealand sourced) and 1% Penicillin-Streptomycin (Fisher Scientific 15140163). Media was changed every 4 days and fibroblasts started to grow out from the tissue at day 4 and 8, respectively. Upon culture to confluency, fibroblasts were trypsinized (TrpLE; Gibco A1285901) and stored in Cryostore CS10 (Stem Cell Technologies 7930) in the vapor phase of liquid nitrogen. Fibroblasts from patients CO1 and CO2 were a generous gift from Dr. Dennis Roop (University of Colorado) and cultured in the above media.

Single step editing/reprogramming: Immediately after transfection of patient fibroblasts with RNPs and ssONDs (see below), cultures were expanded to 225k cells in order to reprogram 3 wells of a 6 well dish. iPS cells were induced with a reprogramming kit a generous gift of iPEACE Inc (Los Altos, CA) according to the manufacturer's recommendations. Briefly, 75k cells were seeded per well of a 6 well plate previously coated with iMatrix (Reprocell NP892-012) and transfected (VFS2205; see above) with 150ng of reprogramming mRNAs (iPEACE Inc) for 10 consecutive days. Cultures were subsequently maintained in StemFit media (Nacalai USA Basic03) according to the manufacturer's recommendations (StemFit was supplemented with basic FGF from Preprotech AF-100-18B and Rock inhibitor from Axon 1683). After emergence of iPS cell colonies, non-reprogrammed cells encasing iPS cell colonies were removed as outlined in Fig. S3A-C and remaining iPS cells were incubated for an additional 24h before manual picking of colonies. Isolated iPS cell colonies were seeded in iMatrix-coated 48 well plates and maintained in StemFit media. After expansion of clonal lines, cultures were duplicated using the procedure recommended by Nacalai USA. gDNA was extracted from 1 duplicate for ddPCR analysis (see above) and sister cultures were maintained for further analysis.

iPS and H9 ES cell differentiation

iPSC lines were maintained in StemFit[®] Basic02 media (Anjinomoto), after expansion the cells were prepared for embryoid body formation using the AggreWell[™] EB 400 microwell plate following the manufacturers recommendations (StemCell Technologies). Cells were dissociated in Accutase (Innovative Cell Technologies) counted and added at 1.2 million cells per microwell in AggreWell media containing 10 μ M ROCK inhibitor (StemCell technologies 72302). Media was carefully changed the following day to remove ROCK inhibition. Embryoid bodies were collected after 48 hours and plated on 10 cm plates at roughly 250 Embryoid bodies per plate in StemFit[®] Basic02 media until cell attachment on vitronectin (Gibco[™]) pre coated plates following the manufactures recommendations. To initiate differentiation of PSCs to

keratinocytes, the PSCs were induced with Essential 6 media (Gibco™) containing 5 ng/mL BMP4 and 1 μ M RA for 7 days; Defined Keratinocyte Serum Free Medium DKSFM (Gibco™) keratinocyte selection media was continued through day 45 in differentiation and continued after MACS enrichment. The enriched keratinocytes were seeded onto Corning PureCoat™ ECM Mimetic 6-well Collagen I Peptide Plate (Corning). H9 ESC were maintained on matrigel hESC qualified matrix (Corning) and in Essential 8 media (Gibco™) until differentiation began same as described above.

ITGA6 enrichment by Miltenyi AutoMACS and CliniMACS separation

Differentiated day 45 cells were dissociated with Accutase (Innovative Cell Technologies) up to 30 minutes, washed, and counted in 10-20 mL of wash buffer containing PBS (Gibco), 1 μ M EDTA (Lonza), 2% BSA (Miltenyi) and 10 μ M ROCK inhibitor (StemCell technologies 72302). For all wash steps, cells were pelleted in the wash buffer at 1000 rpm for 5 minutes. Cell pellets were resuspended in FcR Blocking reagent (Miltenyi), 20 μ L FcR to 80 μ L of wash buffer up to 1×10^7 cells for 5 minutes at room temperature. CD49F biotin antibody (REA518), was added to the blocked cells at 1:50 up for 1×10^7 cells and incubated for 20 min at room temperature. After incubation, the wash buffer was adjusted to 10 mL and centrifuged at 1000 rpm for 5 minutes, then aspirated to completely leaving the cell pellet. Cells were resuspended in 80 μ L of buffer and 20 μ L of Anti-biotin IgG microbeads up to 1×10^7 cells (Miltenyi Biotec) and incubated at room temperature for 15 minutes. Cells were then washed as previously described and resuspended in fresh wash buffer up to 4 mL. The labeled cell suspension was MACS separated using AutoMACS (Miltenyi Biotec) PosselD program setting. For CliniMACS cell enrichment the protocol was modified slightly, cells were labeled as described using the REA518 antibody, the Anti-biotin reagent (microbead) was determined by manufacturers recommendation at 1mL of microbead to 12.5 mL of CliniMACS buffer with 2% HSA, 1 μ M ROCK inhibitor. Program settings were optimized using; 1.1 (gentle), 5.1 (higher purity) and CD34.1 (lower purity, higher viability). Following separation, the cells are resuspended in Defined Keratinocyte Media (Gibco) and plated on ECM Collagen I coated peptide plates (Corning).

Production of RNPs: RNPs were generated by mixing sgRNAs with recombinant CAS9 at a molar ratio of 6:1, followed by incubation at room temperature for 30 minutes. Briefly, to generate 100 μ L with a concentration of 0.5 pmol RNP / μ L we combined 81.13 μ L PBS with 18 μ L sgRNA (650ng/ μ L H₂O) and 0.87 μ L CAS9. CAS9s were sourced from Integrated DNA

Technologies (IDT; Alt-R® S.p. Cas9 Nuclease 1081058 or Alt-R® S.p. HiFi Cas9 Nuclease 1081060) and Aldevron (SpyFi Cas9 Nuclease 9214-0.25MG). sgRNAs were sourced from Synthego and had the following sequences fused to an 80-mer SpCas9 scaffold: C1 GGAUCCACCGUGAGUCCUCG, C2 GGAUCCACCGUGAGUCCUC, C3 CGGGAUCCACCGUGAGUCCU, C4 ACUCACGGUGGAUCCCGCUG, C5 GACUCACGGUGGAUCCCGCU, C6 GGACUCACGGUGGAUCCCGC, and DEB135 ACUGGCACCAUCUCAACCUG.

ssONDs: ssONDs were sourced from IDT and stored as 10uM stock aliquots in H₂O at -20°C. The ssONDs used to edit the Colorado mt allele covered the genomic sequence of chromosome 3 between coordinates 48,570,186 – 48,569,987 (GRCh38.p13) and included the 4 silent mutations described in Fig1B. The ssONDs used to edit fibroblasts of patient DEB135 covered the genomic sequence of chromosome 3 (GRCh38.p13) between the following coordinates and included the 4 silent mutations described in Fig. S5A: ssOND 84bases - 48,572,943 – 48,572,860; ssOND 127bases - 48,573,004 – 48,572,878; ssOND 200bases - 48,572,943 – 48,572,744.

Transfection with RNPs and ssONDs: One day before transfection, 25k fibroblasts were seeded in penicillin-streptomycin-free media in a well of a 24-well plate. Transfection of 5pmol RNP (or of amounts indicated in Fig. S1D) and 10pmol ssOND was performed with VFS2205 transfection reagent (Vivofectamine™ Services from Thermo Fisher Scientific) according to the manufacturer's recommendations.

Extraction of genomic DNA: Genomic (g)DNA was extracted using the Quick DNA miniprep kit (Zymo Research D3025) according to the manufacturer's recommendations. Genomic DNA was eluted and stored in nuclease-free H₂O (Ambion AM9937).

TIDE assay: TIDE assay⁶⁵ was performed as per the inventor's instructions via the online tool at <https://tide.nki.nl/>. Edited loci were amplified from gDNA extracted 3 days (or 7 days for Fig. 4E and G) after transfection with RNPs and ssONDs, using the primers outlined in Table S1 and Q5 Hot-start high fidelity polymerase (New England Biolabs M0494S). PCR products were extracted from standard agarose gels using the QIAquick Gel Extraction Kit (Qiagen 28704) and sequenced by Elim Biopharm (www.elimbio.com).

ddPCR: gDNA was used as a template for droplet digital (dd) PCR according to manufacturer's instructions for the BioRad QX200 system. ddPCR reactions were prepared using 2x ddPCR supermix (BioRad 1863025), bi-allelic reference-HEX primer/probe mix (primer 1: GGATGGGGAATGCAGCTCTT, primer 2: AGTGCGGCAGAATACAGCA, probe: 5'HEX/TGATGGGTT/ZEN/GTGAAGGCAGCTGCACCT/3'IABkFQ), and one of the following FAM-conjugated primer/probe mixes: edited Colorado mt allele-FAM (primer 1: GAGTCAATGAACCTAATGTC, Primer 2: AGAGAGTCCTGGGGTA, probe: 5'6-FAM/AAGGGGGCT/ZEN/CACGGTG/3'IABkFQ), or edited DEB135 mt allele-FAM (primer 1: GACAGAGCTCTTCCCTCTCA, primer 2: CTGCCCCCAGAACACATAC, probe: 5'6-FAM/TGGCCGAGA/ZEN/CGGTGCC/3'IABkFQ). After generation of droplets in the BioRad QX200 generator, which employed DG8 cartridges (BioRad 1864008), gaskets (BioRad 1863009), and droplet generation oil for probes (BioRad 1863005), ddPCR mixes were loaded into 96 well plates (Fisher Scientific E951020346) and sealed with pierceable foil heat seal (BioRad 1814040). PCR reactions were run in a thermocycler using the following parameters: 1x 10 minutes at 95C, 47x 30 seconds at 94C followed by 1 minute 5 seconds at 57C, and 1x 10 minutes at 98C. Subsequently, ddPCR reactions were analyzed in a BioRad QX200 droplet reader and data analysis was performed with BioRad's QuantaSoft Analysis Pro 1.0.596 software. The percentage of edited *COL7A1* alleles was calculated via the following formula: $((\text{concentration in copies per ul of edited Colorado or DEB135 allele [FAM signal]} * 100) / \text{concentration in copies per ul of bi-allelic reference [HEX signal]}) * 2 = \% \text{ heterozygously edited cells}$. For the competitive ddPCR assay detecting the wt and mt (c.1427G>A, p.G476E) allele of the androgen receptor (*AR*), following primer/probe mixes were employed: primer 1: GAAGGCCAGTTGTATGGAC, primer 2: CACATCAGGTGCGGTGAAG, AR-wt-FAM: 5'6-FAM/AGGCGGGAG/ZEN/CTGTAGCCC/3'IABkFQ, AR-mt-HEX: 5'HEX/AGGCGGAAG/ZEN/CTGTAGCCCC/3'IABkFQ. Competitive ddPCR assays were run as above with the following thermocycler parameters: 1x 10 minutes at 95C, 45x 30 seconds at 94C followed by 1 minute 5 seconds at 56C, and 1x 10 minutes at 98C. The percentage of *AR*mt alleles was calculated via the following formula: $((\text{concentration in copies per ul of AR-mt [HEX signal]} * 100) / \text{concentration in copies per ul of AR-mt [HEX] + AR-wt [FAM]}) * 2 = \% \text{ cells with AR mt}$. All ddPCR primer/probe mixes were sourced from IDT as PrimeTime Std qPCR Assays with a primer/probe ratio of 3.6.

Topo cloning: Topo cloning of PCR-amplified *COL7A1* alleles was achieved using the Zero Blunt TOPO PCR Cloning Kit, with One Shot TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific K2800-40) according to the manufacturer's recommendations.

Sanger sequencing: Sanger sequencing was performed at Elim Biopharm according to the provider's recommendations. Sequencing primers for Topo-cloned PCR products had following sequences: Fw 5' GTAAACGACGGCCAG '3, Rw 5' CAGGAAACAGCTATGAC 3'. After purification from standard agarose gels (QIAquick Gel Extraction Kit, Qiagen 28704), PCR products were sequenced with either the Fw and/or Rw primer listed in Table S1.

***in silico* analysis of sgRNAs:** sgRNA sequences were analyzed via the CRISPOR algorithm in order to identify activity and specificity scores and off targets^{66,67}.

PCR: PCRs were performed with the Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs M0494S) and primers outlined in Table S1. *E. coli* colony PCRs (Fig. 1F and Fig. S1F-H) were performed with CloneID 1X Colony PCR Mix (Lucigen 30059-2) and primers outlined in Table S1.

Immunofluorescence microscopy: Cells were fixed (4% paraformaldehyde), permeabilized (0.2% Triton X-100; except for Fig. 2H – TRA-1-81), blocked (BSA), and stained with antibodies against as per standard laboratory procedures. Nuclei were counterstained with DAPI. Images were acquired with a Leica DMI8 inverted microscope. Primary antibodies used: TRA-1-81 (Sigma Aldrich MAB4381), TRA-1-60 (Sigma Aldrich MAB4360), NANOG (Abcam ab21624), K18 (1:800, R&D AF7619), K14 (1:800, BioLegend SIG-3476-100), and p63 (1:100 Gene Tex GTX102425), ITGA6 (1:200, Millipore, MAB1378). Antibodies for mouse grafts comprised of rabbit anti-Involucrin (1:100, abcam), rabbit anti-keratin 14 (1:2000, Covance), mouse anti-keratin 18 (1:800 Abcam), rabbit anti-keratin 10 (1:500, Covance), human specific N terminal anti-collagen VII LH7.2 (1:250 Millipore), The fluorescence images were taken using the TCS SP5 confocal laser scanning microscope (Leica). Tissue sections were co-stained with 1:10000 Hoechst for 10 min. and slides were mounted with the Prolong Gold mounting medium (Life Technologies).

Karyotyping: Karyotyping was performed at WiCell or the Stanford University Medical Center Cytogenetics laboratory after expansion of iPS cells to at least 30 million cells.

Genomic DNA extraction and whole-genome sequencing: Genomic DNA (gDNA) was extracted from cell pellets of skin fibroblasts, iPS cells, and derived keratinocytes from three patients (11 total samples) with the MasterPure Complete DNA Purification Kit (Lucigen #MC85200). The gDNA yield was quantified by the Qubit dsDNA high-sensitivity (HS) fluorescence assay (Invitrogen # Q32851) and ranged from 39.6 to 106.0 ng/uL total in a final volume of 25 uL. The gDNA purity was verified by the NanoDrop 2000 spectrophotometer (Thermo Scientific #ND-2000). Tagmentation-based sequencing libraries were prepared from 500 ng gDNA in duplicate with the Illumina DNA prep (M) kit (Illumina #20018704) using IDT® for Illumina® DNA/RNA UD Indexes Set B, Tagmentation (Illumina #20027214). Libraries were sequenced in 150-bp paired end format on two sequential NovaSeq 6000 S4 lanes (Novogene Corporation Inc.). Each sample obtained at least 40X average coverage after combining the data from both library replicates.

Read pre-processing and variant calling: Raw reads were trimmed of adapter sequences using cutadapt⁶⁸ in pair-end mode and subsequently mapped to the human hg38 reference genome (<https://hgdownload.soe.ucsc.edu/goldenPath/hg38/bigZips/hg38.fa.gz>) using BWA-MEM.⁶⁹ The BAM files were processed by GATK4⁷⁰ to sort mapped reads and mark PCR duplicates, followed by base quality score recalibration (BQSR) which utilized files of known human variant sites recommended in the best practice workflow of GATK4. SNPs and/or Indels were called from processed BAM files using four variant-calling algorithms separately: HaplotypeCaller^{70,71}, Mutect2⁷², Lofreq2⁷³ (SNP only) and Scalpel⁷⁴ (Indel only). The overlapping hits of three SNP callers (HaplotypeCaller, Mutect2 and Lofreq2) and of three Indel callers (HaplotypeCaller, Mutect2 and Scalpel) were regarded as true variants. Functional annotations of the .vcf files were generated using ANNOVAR Wang, 2010, 20601685} with the embedded refGene protocol for hg38. Finally, only variants that do not match the dbSNP database⁷⁵ (build 138) were considered as novel mutations and were included in subsequent analyses.

K-means classification and cell-type specific variant analysis: For each patient-derived lineage, novel mutations called in skin fibroblasts, iPS cells or derived keratinocytes were merged into one table with allele frequency (AF) information retained in all three cell types. Each variant X then has its AF information encoded as vector of three elements:

$$\overline{AF}_X = (AF_{X, \text{fibro}}, AF_{X, \text{iPS cell}}, AF_{X, \text{iSC}})$$

Unsupervised K-means clustering was conducted with all novel mutations using function “kmeans” in R package “stats”. Pseudo seeds were set for the random initiations of clustering centroids to ensure reproducible classification outcomes. Max iteration number was set as 1000 and “Lloyd” was selected as the clustering algorithm. The pre-defined cluster number K was tested from K=3 to K=9 in each individual task. The results of different patients consistently reveal a cluster exhibiting iPS cell/iSC-specific pattern at K=9 as highlighted in suppl Fig S7B. Therefore, we picked the smallest K numbers in each individual that defines such pattern (i.e. K=8 for CO1-131 and CO1-173; K=4 for CO2065(B); K=9 for DEB125-1), and generated variant list from the relevant clusters to be used in suppl Fig S7C.

To further identify variants that are specifically represented in iPS cell and/or iSC compared with their parental fibroblasts, we defined odds ratios OR to measure over-representation of a mutated allele in one cell type relative to another. For example, the odds ratio for variant X to be specifically represented in iPS cell but not the fibroblast is calculated as:

$$OR_{X,iPS\ cell/fibro} = \frac{AF_{X,iPS\ cell} \times (1 - AF_{X,fibro})}{AF_{X,fibro} \times (1 - AF_{X,iPS\ cell})}$$

Any variants X passing the below filters were selected as iPS cell and/or iSC specific variants shown in Fig 4C:

- 1) Shared iPS cell/iSC specific: $(AF_{X,fibro} < 0.25) \text{ AND } (AF_{X,iPS\ cell} > 0.25) \text{ AND } (AF_{X,iSC} > 0.25) \text{ AND } (OR_{X,iPS\ cell/fibro} > 2.0) \text{ AND } (OR_{X,iSC/fibro} > 2.0)$;
- 2) iPS cell specific: $(AF_{X,fibro} < 0.25) \text{ AND } (AF_{X,iPS\ cell} > 0.25) \text{ AND } (AF_{X,iSC} < 0.25) \text{ AND } (OR_{X,iPS\ cell/fibro} > 2.0) \text{ AND } (OR_{X,iPS\ cell/iSC} > 2.0)$;
- 3) iSC specific: $(AF_{X,fibro} < 0.25) \text{ AND } (AF_{X,iPS\ cell} < 0.25) \text{ AND } (AF_{X,iSC} > 0.25) \text{ AND } (OR_{X,iSC/fibro} > 2.0) \text{ AND } (OR_{X,iSC/iPS\ cell} > 2.0)$.

Coverage analysis of potential off-target sites: The 1KB up- and downstream regions of gRNA-targeted site (*COL7A1*) as well as 57 predicted off-target sites were investigated for potential alterations led by gRNA-dependent effects (Fig 4F). Normalized coverages were calculated as absolute read depth per site divided by the mean coverage of entire chromosome.

GO-enrichment analysis: GO enrichment analysis was performed via the online-tool at <http://geneontology.org/>^{76,77}.

STAMP analysis: The Stanford Actionable Mutation Panel of Solid Tumors (STAMP) version 2 sequencing was performed at Stanford University's Anatomic Pathology and Clinical Laboratories.

RNA-seq

Total RNA was isolated following Trizol reagent RNA extraction protocol for cultured cells. The RNA-seq libraries were constructed by TruSeq Stranded mRNA Library Prep kit (Illumina). All the libraries were sequenced to saturation on Illumina Hiseq2000 or NextSeq sequencers.

RNA-seq Analysis

Fastq files were aligned to hg38 using TopHat 2.1.1 with parameters -p 10 --library-type fr-firststrand -r 100 --mate-std-dev 100. Aligned reads were processed to remove PCR duplicates using Samtools 1.8. Raw counts and RPKM values were calculated using HOMER analyzeRepeats.pl. To test for differential expression, raw reads were compared using DESEQ2, and filtered based on an adjusted *p-value* of < 0.05 and 2-fold change.

qRT-PCR

Total RNA was isolated following Trizol reagent RNA extraction protocol for cultured cells. qRT-PCR was performed as described by TaqMan[™] RNA-to-C_T[™] 1-step kit (Applied Biosystems). TaqMan probes available in reagent list.

scRNA sequencing

Differentiated day 7 or enriched and expanded D50 iSCs were dissociated with Accutase (Innovative Cell Technologies) up to 30 minutes, filtered with a 40µm mesh, washed in Wash buffer previously described. Dissociated cells were counted and assayed with trypan blue for live cell counts. Collections with greater than 10% dead cells were processed for dead cell removal using a Dead Cell Removal kit (Mitenyi) following the manufacturers protocol. A total of 10,000 cells were resuspended in Wash buffer at 1,000 cells per µL. Library preparation was carried out following the Chromium Single Cell Chromium Next GEM Single Cell 3' Reagent Kits v3.1 protocol.

Single-cell RNA-seq analysis

FASTQ files were processed using 10x Genomics Cell Ranger v.3.1.0 and the human genome GRCh38. Cells with UMI counts greater than 500 and with mitochondrial percentage below 20%

were included for further analysis. Downstream analyses were performed using Seurat v.4.0.0. 5 iKC samples and a H9KC sample were merged into one object and normalized using the default parameters. To compare between each clone, the Seurat object was split by samples and anchors were identified between samples using FindIntegrationAnchors, followed by integration. A total of 2,000 highly variable features were identified, objects were scaled to regress out cell cycle stages. Cells were clustered using 20 dimensions and a resolution of 0.2 to obtain 8 clusters. FindAllMarkers using $\log(\text{fold-change}) > 0.2$ was used for differential expression analysis within individual clusters. For gene scoring, we used Gibbin dependent gene list from Collier A et al and Holoclone geneset from Enzo E et al to run the AddModuleScore function.

Fluorescence activated cell sorting and Flow cytometry

Dissociated cells were washed with FACS buffer (2% BSA Cat# 130-091-376/1 μ M ROCK inhibitor/ AutoMACS rinsing solution Cat# 130-091-222). After wash steps, cells were fixed and permeabilized with (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Cat # 88-8824-00) then stained for antibodies of interest for 30 min at 4°C (FITC anti-K14, CBL197F from Millipore; PerCP anti-K18 (NB120- 7797, Novus), ITGA6 (PE anti-CD49F BD Cat #555736), all at 1:100 dilution. Enriched iSCs were analyzed using Streptavidin Alexa Fluor™ 647 conjugate (Life Technologies Cat # S32357, 1:500 dilution) and FITC-Labeling Reagent (Miltényi Biotec, Cat # 327806, 1:50 dilution). Composition analysis of enriched iSCs was performed using anti-ITGB4 (BD Cat # 744150, 1:100 dilution) and anti-CD90 (BD Cat # 555595, 1:100 dilution). Cells were strained through 35 μ m mesh. Flow cytometry data was acquired on a BD LSRII in the Stanford Shared FACS Facility with BD FACSDiva Software. Ten thousand events were collected. Analysis was performed with FlowJo software.

***In vitro* skin reconstitution assay**

Generation of organotypic epidermis was performed by following the protocol described previously⁸ with minor modifications. Derived induced keratinocytes and unsorted passaged differentiated cells were expanded in Defined Keratinocyte SFM (Gibco™) until confluent then passaged onto devitalized human dermis seeded at a density of 1×10^6 cells. The medium was then gradually changed to 7F stratification media for 7 days, after which the dermal sheet was raised to the air-liquid interface. After 2 weeks, the reconstituted epidermis was collected for IF staining.

Mouse skin engraftment with iPS cell derived iSC and Luciferase-RDEB-SCC lines

All animal experiments followed the NIH (National Institutes of Health) *Guide for the Care and Use of Laboratory Animals* under Stanford APLAC (Administrative Panel on Laboratory Animal Care). Xenograft protocol was performed as described previously^{8,48}. ITGA6-enriched cells (1×10^6) were seeded onto a 1.5-cm² piece of devitalized human dermis (New York Firefighter Skin Bank) and grown in DKSFM (Gibco™) for 10 days, followed by Keratinocyte Growth Medium (Gibco™) for 5 days. Next, the pieces were grafted onto the backs of NSG mice for 1-6 months. Upon collection, the pieces were embedded in OCT and paraffin for immunofluorescence analysis. In vivo tumorigenicity of the reprogrammed and re-differentiated EB-SCC-iSCs expressing the luciferase gene, were created as previously described⁵¹ were spiked with normal human keratinocytes NHKs to generate the 3D skin constructs and then grafted onto immunodeficient mice. Tumors were formed in mice grafted with RDEB-SCC-iSCs within 4 weeks and detected with the bioluminescence signal intensities.

Alu-qPCR

In grafted mice, detection of emanating cells using Alu-sequence was performed on mouse organs including lymph nodes, liver, lungs, spleen, kidneys, heart, brain, and blood. The organs were resected and transferred to a sterile petri dish containing cold PBS, washed, finely minced, and weighed. DNA was extracted from the minced tissue according to the QIAamp DNA mini kit protocol (Qiagen Cat# 51304) with an overnight cell lysis at 56°C and 10-minute lysis for 100 uL blood. Alu-qPCR was performed using 10ng of extracted DNA in triplicate using amplification protocol by Applied biosystems (Universal Master Mix II, no UNG: Applied Biosystems Cat# 4440040 and Alu Probe: ThermoFisher Scientific Cat# 4351372).

FIGURES AND LEGENDS

Figure 1: Optimized editing of the *COL7A1* Colorado mutation (7485+5 G>A). (A) Overview of single editing&reprogramming manufacturing step. (B) Overview of the Colorado mutation (red) and (+) or (-) strand ssODNs used for gene-editing. 6 possible PAM sites (C1-6) can be engaged via sgRNAs to mediate cutting of pathogenic alleles via CRISPR/CAS9. ssODNs encode for 4 silent mutations (blue) and the wild type sequence (green). (C) Absolute CRISPR/CAS9 cutting efficiencies of the homozygous Colorado mt in CO2 patient fibroblasts as mediated by sgRNA C1-C6 (n=2; stdev). (D) Relative CRISPR/CAS9 cutting efficiencies of the homozygous (CO2), heterozygous (DEB125) Colorado mt or the wild type locus as mediated by sgRNA C2 or C4 in indicated patient fibroblasts (n=2; stdev). (E) *COL7A1* editing efficiencies measured by ddPCR in CO2 primary patient fibroblasts after transfection with ssODNs and sgRNA/CAS9-containing RNPs as indicated. A bi-allelic locus (green) is used as a reference for calculating *COL7A1* editing (blue) efficiencies. Both, strandedness of the ssODN and sgRNA affect editing efficiencies. Ctrl omitted sgRNAs. (F) Agarose gels visualizing 77 *E. coli* colony-PCRs to detect edited Topo-cloned *COL7A1* alleles from cells treated as in (E) with ssODN/sgRNA combinations as indicated (see Fig. S1 for remaining ssODN/sgRNA combinations). A primer specific for intended silent mutations (see Fig. 1B) only yields PCR products of alleles with integration of donor sequences (asterisks). (G) Summary of *COL7A1* editing efficiencies achieved with different ssODN/sgRNA combinations as measured by ddPCR (Fig. 1E) and as observed by Topo-cloning of individual alleles (Fig. 1F). Mono-allelic integrations of ssODN sequences were assumed for calculations (see discussion). (H) Sanger sequence traces of unedited and edited alleles from (Fig. 1F, S1H). Asterisks indicate integration of intended silent mutations (blue) and repair of the pathogenic mutation (green).

Figure 2: Successful single manufacturing step editing/reprogramming of patients CO1, CO2 & DEB125. (A) *COL7A1* editing efficiencies as measured by ddPCR in CO1 and CO2 primary patient fibroblasts (homozygous Colorado mt) after transfection with (+)ssODN and RNPs containing sgRNA C4 and high fidelity CAS9 HiFi or SpyFi as indicated. A bi-allelic locus (green) is used as a reference for calculating *COL7A1* editing (blue) efficiencies, assuming mono-allelic editing events. Ctrl omitted sgRNAs. (B) Reproducible *COL7A1* editing in CO2 primary patient fibroblasts as in (Fig. 2A) with SpyFi CAS9 (error bars are sem). (C) Representative ddPCR screen of 64 single-step edited/reprogrammed iPS cell lines derived from patient CO1 fibroblasts. Ratios of signals detecting edited *COL7A1* alleles (blue) and a bi-allelic reference locus (green) are used to identify mono- (0.5 +/-0.19) or bi-allelic (1.0 +/-0.19)

editing events (black values; red values below/above cut off indicate potentially mixed or incorrectly edited clones). **(D-E)** Agarose gel visualizing PCR amplicons of a 731bp (D) and 2418bp (E) sequence surrounding the edited *COL7A1* locus from single-step edited/reprogrammed iPS cell lines derived from 3 patients heterozygous (DEB125) and homozygous (CO1 and CO2) for the Colorado mt. Note that some samples yield 2 PCR products, indicative of large InDels on one of the *COL7A1* alleles. InDels can be substantial (e.g. line CO2-65(B)), so that they are only included on bigger PCR products (E). See text for details. **(F)** Sanger sequencing of the smaller PCR product from line CO2-65(B) from (Fig. 2E) reveals a large 654bp deletion when aligned with the wt *COL7A1* sequence. This deletion is not detected by smaller PCR amplicons in (Fig. 2D). **(G)** Summary of single-step editing/reprogramming screens conducted with sgRNA C4 and (+)ssODN from 3 patients as indicated (top). Topo-cloning and sanger sequencing of PCR products from (Fig. 2D-F) confirms correct *COL7A1* editing on target alleles in 15 of 17 single-step edited/reprogrammed iPS cell lines. **(H)** Immuno-fluorescence microscopy images of single-step edited/reprogrammed iPS cells and parental fibroblasts stained for pluripotency markers TRA-1-81 and NANOG from 3 patients. DAPI visualized DNA. **(I)** Summary of flow cytometry analysis of single-step edited/reprogrammed iPS cells from 3 patients for CD90 and the pluripotency marker TRA-1-60 (see Fig. S3D for details).

Figure 3: Generation of organotypic skin grafts at clinical scale **(A)** Differentiation strategy and cell enrichment with AutoMACS pro-separator for iPS cell clone comparison following a defined GMP compatible protocol to manufacture induced skin composite (iSC) grafts. **(B)** UMAP cluster representation of scRNA sequencing of H9 ES cell differentiation at Day 7 reveals the three lineage clusters: ectoderm (orange), mesoderm (blue) and neuroectoderm (green). **(C)** Violin plots depicting RPKM relative expression of representative ectoderm, mesoderm and neuroectoderm genes. **(D)** Immuno-fluorescence (IF) of iPS cell line DEB135-10-derived iSCs, stained for p63, K18 and K14 at day 45 of differentiation as in (Fig. 3A). **(E)** ITGA6 expression as per RNA-seq in H9 ES cells during iSC differentiation. Time points are indicated (D: day). NHKs were used as a positive control. **(F)** IF staining of AutoMACS-ITGA6 enriched, expanded iSCs. p63 (Cyan), K18 (red), and K14 (yellow). **(G)** Flow cytometry of day 45 unsorted H9 ES cells. Cells highly double positive for ITGA6 PE (y-axis) and K14 FITC (x-axis) are in high gate (red) and lower ITGA6/K14 expressing cells are in low gate (blue) to evaluate expression of K18 (PER-CP) in the subpopulations (right). **(H)** (Top left) Bright field image of FACS sorted ITGA6^{HI} H9 ES cell-derived iSCs expanded to day 50 and used for organotypic stratification (Top right).

IF shows normal polarization and stratification of the epidermis from the ITGA6^{Hi} H9 ES cell-derived iSCs (Collagen 7 (green), Involucrin (red), DAPI (blue)); (Bottom left) bright field image of unsorted H9 ES cell derived iSCs used for organotypic stratification (Bottom right). IF of corresponding unsorted H9 cells revealed disorganized layering and stratification. **(I)** Flow cytometry analysis of %ITGA6 positivity measured before and after AutoMACS enrichment for 5 differentiated iPS cell patient lines (independent differentiation runs for each clone n=4-10). **(J)** % Coupling efficiency (CE) determined by the equation %CE= live sorted iSCs/iPS cell input*100. **(K)** UMAP showing overlay of scRNAseq datasets from (Fig. 3L) with color scheme as indicated. 4 ectoderm (C1, C2, C4, C7), 3 mesoderm (C3, C8, C5) and 1 melanocyte / neuroectoderm-derived (C6) clusters were identified. **(L)** UMAP of scRNA sequencing of 5 individual patient iSCs and H9 ES cell derived iSC after ITGA6 enrichment and expansion for 2 passages *in vitro*, revealing 8 clusters (C) comprising the DEBCT product. Gibbin dependent clusters are outlined in dotted lines (see text for details), and arrows show lack of these clusters in products derived from iPS cell lines C01-131/173.

Figure 4: Genomic and chromosomal stability of single manufacturing step

edited/reprogrammed iPS cells and iSCs. **(A)** Normal karyotypes were observed in 11 of 12 single-step edited/reprogrammed iPS cell lines from 5 patients (CO1, CO2, DEB125, DEB134, and DEB135). **(B)** K-means clustering of all novel variants (n=111741) found by 40x whole genome sequencing (WGS) in fibroblasts and thereof derived iPS cells and iSCs from patient DEB125 (see Fig. S7 for other patients). Only a small sub-set of variants (red frame) is found to have differential allele frequencies (AFs) in iPS cells/iSCs compared to parental fibroblasts. **(C)** A defined cut-off (AF=0.25), combined with odds ratio filtering (see material and methods) identifies variants from 40x WGS (red: SNPs; green: InDels) specifically found in cell types from patient lines as indicated. Grouping of variants present in cell lines derived from 3 patients in Venn diagrams indicates absence of positive selection for any mutations. The majority of identified cell type specific variants is found in intergenic or intronic sequences (pie charts). No gene ontology (GO) term enrichment was found in identified variants. **(D)** Aligning all shared iPS cell/iSC-specific variants identified by k-means clustering (Fig. 4B, S7B) and AF-cut-off filtering (Fig. 4C) with the used seed sequence of sgRNA C4 within a 25bp search radius that must contain a NRG PAM-motif does not identify any significant homologies. The outlier (circles) with the most similarity to sgRNA C4 still exhibits 6 mismatches (see text for details). **(E)** TIDE analysis of sgRNA C4-mediated CAS9-cutting in CO2 patient fibroblasts at the *COL7A1* on-target (top) and the most likely *in silico* predicted off-target, i.e. *NOTCH1* (bottom).

Measurements were taken 100bp-50bp upstream (i.e. internal Ctrl) and 10-60bp downstream of the predicted cut sites. **(F)** Plots of normalized WGS coverage 1000bp up-/downstream of the *COL7A1* on-target (top) and the *NOTCH1* off-target (bottom) from fibroblasts and thereof derived iPS cells as indicated. Note the sharp drop of coverage at the *COL7A1* locus in iPS cells due to a heterozygous deletion of 654bp (see Fig. 2D-G). **(G)** Summary of (Fig. 4E-F) for all *in silico* predicted exonic and intronic off-targets and the *COL7A1* on-target (left column). For TIDE analysis (middle column), noise-signals from internal controls were subtracted from values measured at cut sites (Fig. 4E). No InDels were found at any off-target in 4 clonal iPS cell lines from 3 patients (Fig. 4F). Heterozygous 1bp, 10bp (not shown) and 654bp big deletions were detected on *COL7A1* on-target alleles of patients with homozygous mutations (i.e. CO1 and CO2). **(H)** Variants identified via the STAMPv2 oncopanel from fibroblasts and thereof derived iPS cells and iSCs as indicated. Germline variants (green) are found in all 3 cell types. A heterozygous mutation of the androgen receptor (red) stems from clonal expansion of a fibroblast sub-population (3%) already carrying this lesion. Blue indicates variants identified by secondary methods (see text and Fig. S7F-H) for details.

Figure 5: Patient iPS cell-derived organotypic skin grafts survive in mice with a favorable safety profile. **(A)** Table of mouse graft success determined by IF staining of basement marker Collagen 7 (COL7) and granular marker involucrin (IVL) at 1 month. Graft attempts include at least two distinct manufacturing runs **(B)** Representative IF image of 1 month DEB-125-1 derived iSC graft. Keratin (K)14, K10, involucrin (red), Collagen 7 (ColVII; green), DNA (blue). **(C)** Quantification (%) of 8 different cell type clusters (C1-8) comprising the DEBCT product and identified via individual scRNA sequencing in. **(D)** Strong positive correlation between % graft success rate in (Fig. 5A) and average Gibbin-dependent gene expression as quantified by scRNA sequencing. **(E)** Representative flow cytometry of cell composition of H9 ES cell derived iSCs labeled for ITGB4 and the Gibbin-dependent fibroblast marker CD90/Thy1 before grafting on to mice. **(F)** Bar graph of flow cytometry quantifying the average expanded iSC cell composition prior to grafting. **(G)** qRT-PCR detection of pluripotent marker expression (LIN28A, NANOG, OCT4 and SOX2) in the iSC product. H9 ES cells and TERT keratinocytes (KC) were used as controls. **(H)** Table summarizing Alu-qPCR-based biodistribution and histology-based tumor detection results from mice at 1-, 3- and 6-month post iSC grafting. **(I)** Diagram of method for detecting evading iSCs into mouse organs using human Alu-qPCR. **(J)** Representative Alu-qPCR from organs of DEB-125-1 derived iSC grafted mice at indicated time points. LOD is level of detectability in tissues spiked with human DNA from TERT keratinocytes.

SUPPLEMENTARY FIGURES AND LEGENDS

Figure S1: Optimized editing of the Colorado mt (7485+5 G>A). **(A)** *In silico* predicted efficiency (middle column) and specificity (right column) scores^{66,67} of the 6 tested sgRNAs (left column) used to mediate CAS9-cutting of the pathogenic Colorado allele. **(B)** Agarose gel visualizing PCR amplicons of a 731bp sequence surrounding the *COL7A1* target locus from homozygous CO2 patient fibroblasts transfected with RNPs containing CAS9 and indicated sgRNAs (Ctrl. omitted sgRNA). Replicate experiments are shown. **(C)** TIDE traces of PCR amplicons shown in (Fig. S1B). **(D)** *COL7A1* editing efficiencies as measured by ddPCR in DEB125 primary patient fibroblasts (het. Colorado mt) after transfection with ssODNs and sgRNA/CAS9-containing RNPs as indicated. A bi-allelic locus (green) is used as a reference for calculating *COL7A1* editing (blue) efficiencies. Ctrl omitted sgRNAs. **(E)** InDel formation in CO2 fibroblasts after transfection with indicated amounts of sgRNA C4-containing RNPs as measured by TIDE (n=2; stdev). Ctrl. omitted sgRNA. **(F-H)** Agarose gels visualizing 78 E. coli colony-PCRs to detect edited TOPO-cloned *COL7A1* alleles from CO2 fibroblasts transfected with ssODNs and RNPs containing sgRNAs as indicated. A primer specific for silent mutations (Fig. 1B) only amplifies PCR products of alleles with integration of donor sequences (asterisks).

Figure S2: Single manufacturing step/editing reprogramming of patient DEB125 with the less specific sgRNA C2. **(A)** We assessed combined repair and reprogramming using sgRNA C2 and (+)ssODN in fibroblasts from patient DEB125. RNPs and the ssODN were transfected, followed by 10 consecutive daily doses of reprogramming mRNAs. This treatment yielded 74 iPS cell colonies. Screening via ddPCR, identified 9 candidate lines (~12%) with potential mono- or biallelic integration of ssODN sequences at the *COL7A1* locus. This is consistent with the expected ~15% *COL7A1*-editing frequency (Fig. S1D). Ratios of signals detecting edited *COL7A1* alleles (blue) and a bi-allelic reference locus (green) are used to identify mono-allelic (0.5 +/-0.19) or bi-allelic (1.0 +/-0.19) editing event (black values; red values below/above cut off indicate potentially mixed or incorrectly edited clones). **(B-C)** We established 5 of these lines and used their genomic DNA to further characterize the edited *COL7A1* locus. Agarose gel visualizing PCR amplicons of a 731bp (Fig. S2B) and 4560bp (Fig. S2C) sequence surrounding the edited *COL7A1* locus from established single-step edited/reprogrammed iPS cell lines from (Fig. S2A). **(D-E)** Cloning of the PCR amplified region followed by Sanger sequencing revealed that 2 of these 5 lines had correctly edited *COL7A1* alleles, displaying all intended silent

mutations and a wt sequence at the Colorado mutation site. Shown is the summary of single-step editing/reprogramming screen conducted with sgRNA C2 and (+)ssODN from patient DEB125 (top). Topo-cloning and sanger sequencing of PCR products from (Fig. S2B-C) identified 2 iPS cell lines with correct donor integration (i.e. 125-C2-39 and 125-C2-69) that however, occurred on the wrong allele carrying the second heterozygous compound mutation of this patient (i.e. Mexico mt; 6527dupC). This is in agreement with the low specificity of sgRNA C2 for the Colorado-allele (Fig. 1D). We note that sgRNA C2 is capable of targeting the disease allele as we have successfully used it to derive such cell lines from fibroblasts of patient CO2 (homozygous for the Colorado mt; Fig. 4A).

Figure S3: Efficient derivation of high-quality iPS cells from primary RDEB patient fibroblasts.

(A) Phase contrast microscopy pictures of cell cultures during single-step editing/reprogramming from time points (D: day) and patients as indicated. iPS cell colonies emerge around D11-14. **(B)** Non-reprogrammed cells grow to confluency, adhere together, overlay iPS cell colonies and can be mechanically removed. **(C)** iPS cell colonies remain after removal of non-reprogrammed cells. **(D)** Flow cytometry analysis of single-step edited/reprogrammed iPS cell lines with and without labeling (antibodies; abs) of the pluripotency surface marker TRA-1-60 as indicated.

Figure S4: ddPCR screening of single-step edited/reprogrammed iPS cells. (A-C) Screen of 122 (A), 293 (B), and 24 (C) single-step edited/reprogrammed iPS cell lines via ddPCR from patients as indicated. Ratios of signals detecting edited *COL7A1* alleles (blue) and a bi-allelic reference locus (green) are used to identify mono-allelic (0.5 ± 0.19) or bi-allelic (1.0 ± 0.19) editing events (black values; red values below/above cut off indicate potentially mixed or incorrectly edited clones).

Figure S5: Optimized single manufacturing step editing/reprogramming of patient

DEB135 (6781C>T). **(A)** Overview of the *COL7A1* target mutation 6781 C>T (red) and (+)/(-) ssODNs used for editing. PAM site to mediate cutting of pathogenic allele via CRISPR/CAS9 is shown. ssODNs encode for the wild type sequence (green) and 4 silent mutations (blue) used for detection of editing events. **(B-C)** *COL7A1* 6781C>T editing efficiencies as measured by ddPCR in DEB135 primary patient fibroblasts after transfection with ssODNs of various lengths, strandedness, and sgRNA 135/CAS9-containing RNPs as indicated. A bi-allelic locus (green) is used as a reference for calculating *COL7A1* editing (blue) efficiencies, assuming mono-allelic

integration of donor sequence. The length of ssODNs correlates with editing efficiencies. Ctrl omitted sgRNAs. **(D)** Screen of 83 iPS cell lines via ddPCR after single-step editing/reprogramming of fibroblasts from patient DEB135 with (-)ssODN and sgRNA 135. Ratios of signals detecting edited *COL7A1* alleles (blue) and a bi-allelic reference locus (green) are used to identify mono-allelic (0.5 +/-0.19) or bi-allelic (1.0 +/-0.19) edited iPS cell lines (black values; red values below/above cut off indicate potentially mixed or incorrectly edited clones). **(E)** Agarose gel visualizing PCR amplicons of a 696bp sequence surrounding the edited *COL7A1* locus from established iPS cell lines from (Fig. S5D). Unedited fibroblasts were used as Ctrl. **(F)** Sanger sequencing traces of PCR amplicons shown in (Fig. S5E) containing both *COL7A1* alleles. Unedited fibroblasts show the heterozygous 6781C>T mutation as a double peak. Correctly edited iPS cell lines show heterozygous integration of intended silent mutations (blue asterisks) and repair of the pathogenic mutation (green asterisks). iPS cell line 135-31(A) only displayed the wild type sequence, indicative of a large InDel prohibiting amplification of the target allele. **(G)** Summary of single-step editing/reprogramming screen of patient DEB135 fibroblasts (top). Sanger sequencing of individual Topo-cloned alleles (Fig. S5E) confirmed correct editing in 3 iPS cell lines. See text for details. **(H)** STAMPv2 oncopanel sequencing of indicated cell lines did not identify any variants. **(I)** Immuno-fluorescence microscopy images of DEB135 fibroblasts and thereof derived iPS cells. NANOG (red), TRA-1-60 (green), and DNA (blue).

Figure S6: Efficient ITGA6 enrichment using CliniMACS purification for clinical scale manufacturing. **(A)** qRT-PCR of pluripotency marker expression of iPS cell lines at Day 0. TERT keratinocytes (KC) were used as the negative control. **(B)** Principal component analysis of RNA-seq from the CliniMACS ITGA6-enriched and reduced iSC cells compared to a H9-ES cells differentiation time course (D0-D45); H9 keratinocytes (KC) and NHK (positive control). **(C)** RNA-seq expression of CliniMACs (CM) enriched and reduced populations compared to H9 Day 0-45 differentiations, H9 iSC cells, and NHKs, illustrating the non-target population markers (TRA-1-60, Thy1/CD90, KRT18) and the target population markers (P63, KRT14 and ITGB4). **(D)** Table of optimizations for CliniMACs including program and reagents changes for each CM run with pre and post enrichment cell counts. **(E)** % ITGA6 positive cells of CM-sorted populations compared to the unsorted population by flow cytometry. **(F)** %Coupling efficiency (CE) determined by the equation %CE= live sorted iSCs/iPS cell input x 100 for CliniMACS runs CM 1-5. Note efficiency of CD34.1 automation program (i.e. CM 5). **(G)** Number of both viable and non-viable CliniMACS enriched iSCs. **(H)** Flow cytometry plots and corresponding bright

field images of iSC Enriched and Reduced Fractions from CM3 CliniMACS separation (10x Magnification). **(I)** Violin plots of relative expression levels of key marker genes representing each cell cluster contained in the iSC product (Fig. 3), including basal keratinocytes (C1), cycling holoclones (C2), early differentiating keratinocytes (C4), Gibbin-dependent mesoderm (C5, C8), pre-vascular mesoderm (C3), and melanocytes (C6).

Figure S7: Genomic and chromosomal stability of single step edited/reprogrammed iPS cells and iSCs. **(A)** Representative normal karyotypes of four single-step edited/reprogrammed iPS cell lines from 3 individuals. **(B)** K-means clustering of all novel variants found by 40x whole genome sequencing (WGS) in fibroblasts and thereof derived iPS and iSC cells from patients CO1 and CO2 (CO2-65(B) n=114594; CO1-131 n=102278; CO1-173 n=101915). The red frame highlights differentially expressed allele frequencies (AFs) of variants in iPS/iSC cells compared to parental fibroblasts. **(C)** Variants (red: SNPs; green: InDels) that are specifically found in iPS and iSC cells of indicated cell lines as identified by k-means clustering. Grouping of variants in Venn diagrams indicates virtually no selection for mutations. Variants of clusters (cl.) showing clear separation between AFs found in fibroblasts and AFs found in iPS/iSC cells from feature spaces with the lowest k (indicated) were selected for this analysis. **(D)** AF cut-off filtering and k-means clustering identify virtually the same (>89%) variants specific to iPS/iSC cells. **(E)** Percentage of identified cell type-specific variants with no reads in other cell types as indicated. Note that virtually all iPS cell-specific variants identified by AF cut-off filtering (Fig.4C) were also found at lower AFs in other cell types. **(F)** Sanger sequencing traces of a PCR amplicon containing the androgen receptor (AR) locus c.1427 from indicated iPS cell lines of patient CO2. A heterozygous mutation was found in 4 of 11 iPS cell lines (double peaks). **(G)** A competitive ddPCR assay with probes specific for the wt and mt AR sequence from indicated cell lines confirms that 4 lines derived from patient CO2 harbor the mutation. Note that ddPCR detects this mtAR also in CO2 fibroblasts at low frequencies. **(H)** Sanger sequencing traces of a PCR amplicon containing the CDKN1B locus c.426 confirm a heterozygous germline mt (double peak) present in fibroblasts and thereof derived iPS/iSC cells of patient DEB125. Fibroblasts of patient CO1 were used as a negative control.

Figure S8: iPS cell detection assay, and qualification of human-specific Alu-PCR and tumor detection assays. **(A)** qRT-PCR of LIN28A to detect remnant iPS cells in expanded ITGA6-enriched cultures. **(B)** Schematic of assay to detect luciferase expressing-RDEB-

squamous cell carcinoma (SCC)-cells in iSC cell grafts from a 4-week mouse tumor model. Bioluminescent detection of NHKs alone, 10, 100, 250 and 750 RDEB patient-derived SCC cells in 4-week-old grafts on mice to determine a level of detection (LOD) and **(C)** corresponding H&E histology of graft site. **(D)** Alu-qPCR detecting TERT-human keratinocytes in mouse cells from indicated organs to determine the level of detection (LOD) and quantification (LOQ) of human DNA for the biodistribution assay (SEM). **(E)** Alu-qPCR experiments performed on organs and blood of iSC grafted mice. DEBCT product from iPS cell clones as indicated and the time on the mouse (1-9 months). At left of each graph are the positive controls, with the tissue sampled on the X axis. Error bars are +/- SEM.

Table S1: PCR primer sequences

target	primer 1	primer 2	size
Colorado mt allele (Fig1C&D, suppl. Fig1B-D, Fig2D, suppl. Fig2B, Fig4E&G)	TAGGTGGGACAAGTGCTGCTGACTC	CCTTTAGTCCTGCACTCCCAACATCAC	731bp
Colorado mt allele big (Fig2E)	TCATGTCTGAGCTCCTGTGAGCC	TCCACAGACTGGCTCATTTCTCACC	2418bp
Colorado/Mexico mt allele (suppl. Fig2C)	GCGTGGTATGGCTGGGCCTGAAG	CCTTTAGTCCTGCACTCCCAACATCAC	4560bp
DEB135 allele (suppl. Fig5E&F)	CTGACTGGACCTACTGGAGCTGTG	CCTGTGGGGAATGCTAGTGAGTTTCC	696bp
E. coli colony PCR (Fig1F & suppl. Fig1F-H)	GGACAAGTGCTGCTGACTCT	CACCGTGAGCCCCCTT	327bp
AR (suppl. Fig6F)	CTCGCATCAAGCTGGAGAAC	ACACATCAGGTGCGGTGAAG	354bp
CDKN1B (suppl. Fig6H)	CTTGGAGAAGCACTGCAGAGAC	CGAAAAGCAAGCTAAGGTTAACACC	426bp
<i>in silico</i> predicted off-targets (Fig4E&G)	primer 1	primer 2	size
NOTCH1	GTGTGACGCAGCCTGTGGGTGC	AAGCGCACCAGTTCTTCAGGACAGAC	648bp
AC144450.2	GAGCTTGCACTGAGCAGAGATCG	CCTCTTCCCTGACTTCTTACACTG	727bp
PAK4	CAGAGAAGAGGCCCAAGTCTTCC	GCACCTCGTTGAAGAGCAGCTC	835bp
CAPN12	GCATTGAGTTCTTCTCTAGTCC	GTAATTTGCAGGGAGATCGACGAC	702bp
FXYD1/CTD-2527I21.4	GAGGAACTAAGGCACAGGAGG	CCTTCAACTCTGGCACTTGGCC	763bp
HS3ST4	CGTGACCAGGGCCATCTCTG	CCAGATGCTGCTGCCAGATGG	723bp
MARS	GGAATAGGGCAGAGCCTTGGG	GAGATCAGCAAACGGGACCTAGC	357bp
PAQR7/RP1-125I3.2	CCCACATAGTCCAGGAAGAAGAAGC	CCTCTCATGTGTTTCATATGGCCAG	829bp
RP11-334A14.8/SLC1A7	CTGGGCTTTCTGCCTACCAGTG	CCATTTCTCTGCGCTGCACAATCC	773bp
RP5-1159O4.1/MIOS	CGACAGAGTGGTTCTGAGAAGC	GGGTACAACGGGAACAGGGCA	746bp
SLC25A29	AGCACTGGTGTCCCATCTGCAG	GTTTGTACTCAGCAGATGCCAGCTG	751bp
UBE2I	CAGTCCCTCACACACACAC	AGGTCTCGGGAATCTGCTTT	719bp
ZNF385A	GCATCCTAGTTTCCAGCTTCGTCC	CCCAGAAGGATGTCGGAGTATC	767bp
FAM207A	CAGAGTCAAGGGTTAGAGG	GACAGGCTTCTTCTCTGTGCCCT	705bp
LINC00710	CAGAGCAGCCAGTCCAAAGACC	GGCCACACTGATAAGGTGGAGAC	776bp
OLFM1	CCATGGATCCCCTAATCCAAATGCC	TGAGAGGAACAACGCCCTCCTGG	760bp
PADI3	CGTGATAAGAGTGCAAGGCTGG	GCAGCTCCCTCCACTCTTACAAG	825bp
PLXNA1	GTCATGGACTGCCCCAATCAGC	GCACGGGTCTAGAATGTTCCTAGT	714bp
PRR26	CGGAGATTCTAGCCCTTGTCCTG	CCAACACTGTGCGTGTGACTCAG	746bp
RBM10	CTGAGCTCAAGCAGTCTCTACG	CCATTTACTCGGCAGGGACAGTC	558bp
TIMM44	CAGCCTCCTCAGAAAACAGCCTCG	GTCTGTGAGGACTGCTAGAGGC	741bp
UST	GTCCAGGGCACCTGTAAGTAAGCC	GGGTGTCAATGCACGACATTCTCCC	766bp
VWA3A	TAGGCAGGAGGTCCCAACAGC	GCTAGTCCTTCTCAAGCCCCTTC	653bp
VWF	TAGTCACTGGCTGGCTGGGTGTG	ACAGCATTCCTGGACTCTGCAGCC	709bp
ZBED4	GGCTCCTGCTGGAATTTGTGGCAG	GGGCCAGTGAGCAAAGTCGCATC	744bp
ZNF534	CTGAAATGCCAGGCATTGGAGTTGC	GCAGCAGATCACAGGTGCTGAGC	868bp

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Fig1

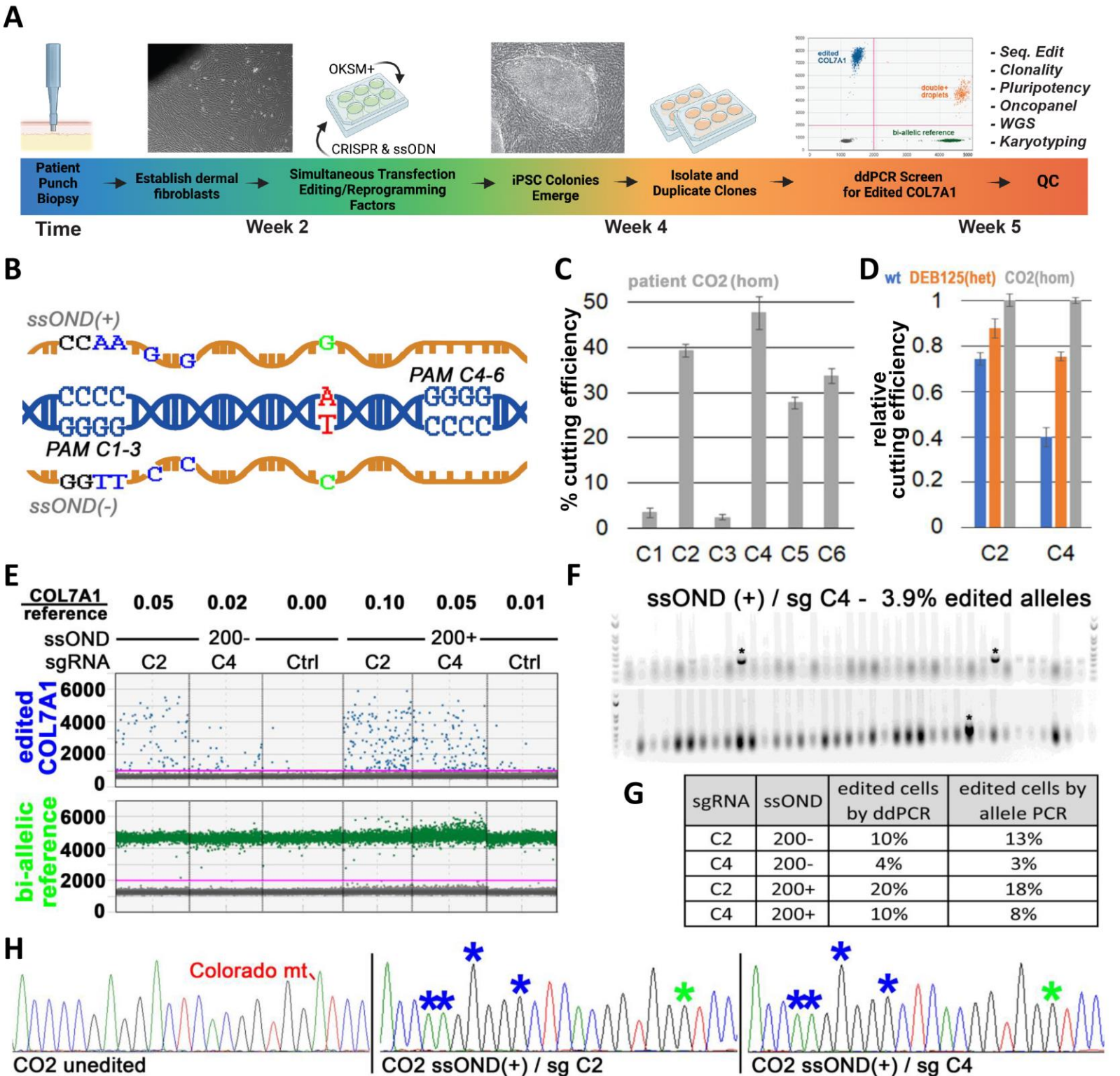
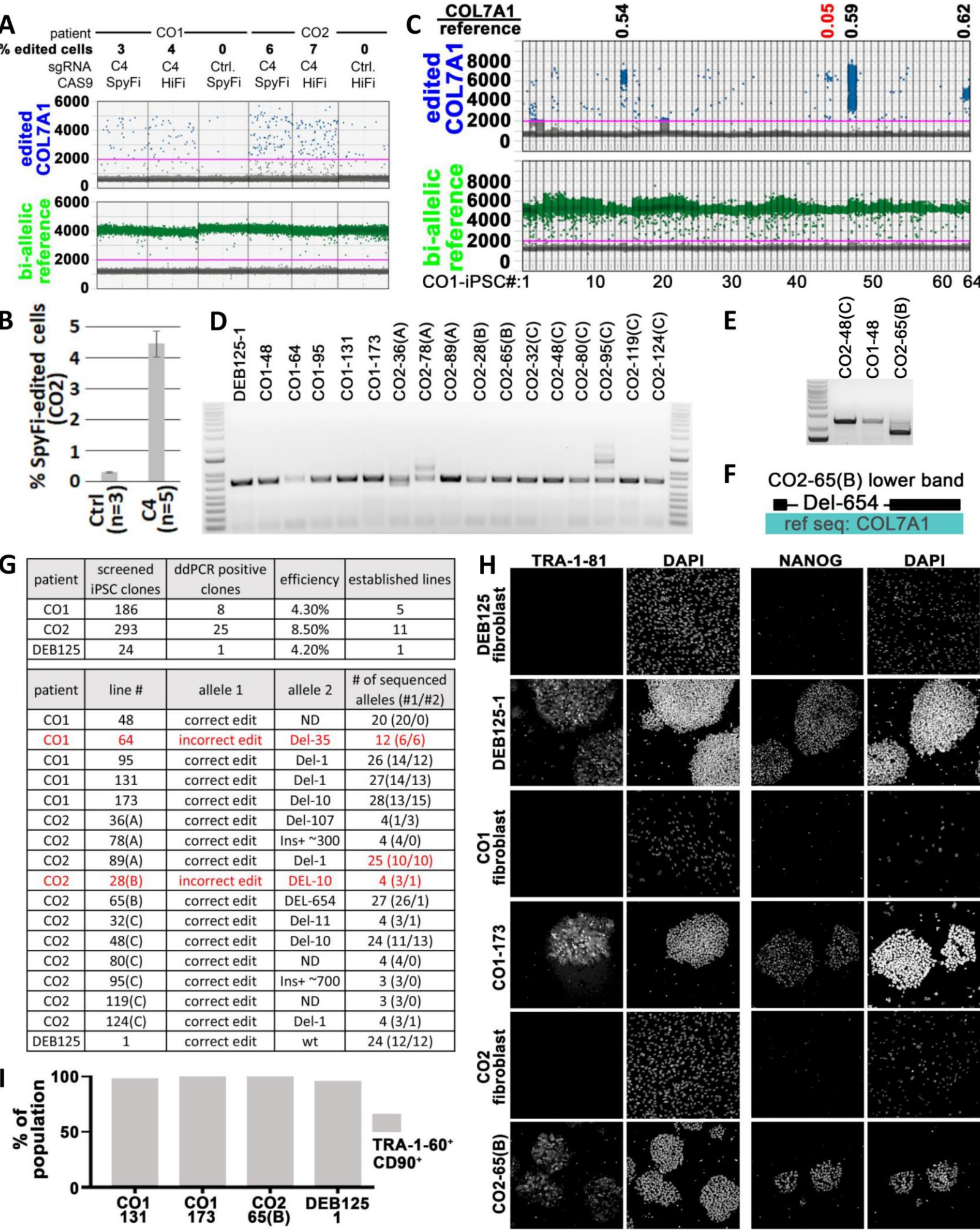


Fig2



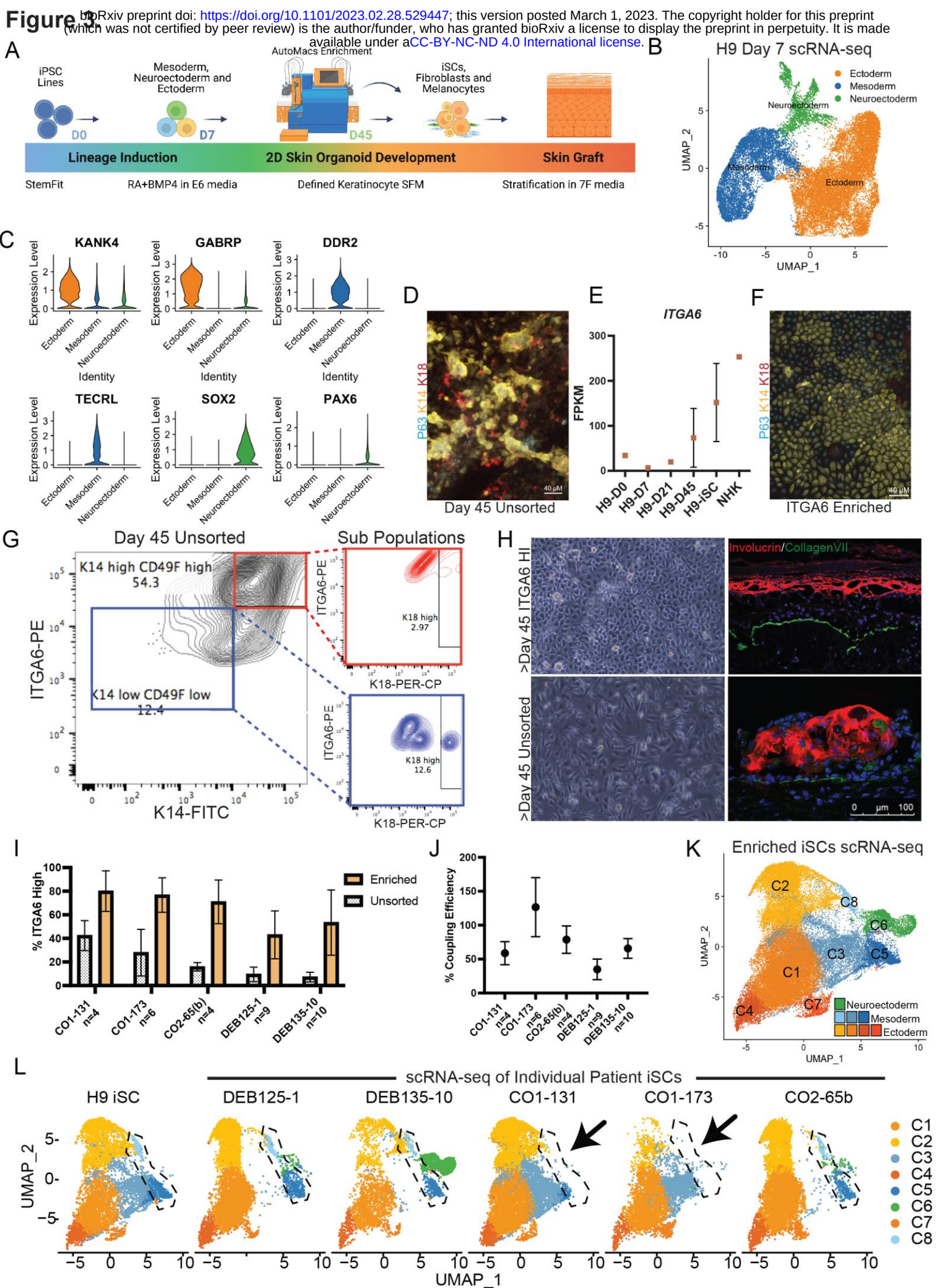
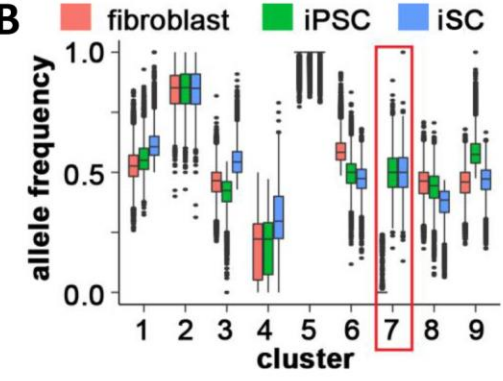


Fig4

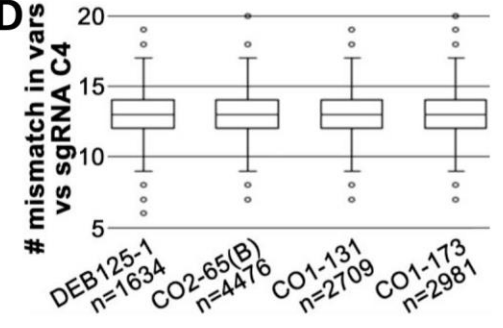
A

line	sgRNA	karyotype
CO1-131	C4	normal
CO1-173	C4	normal
CO2-36(A)	C4	normal
CO2-65(B)	C4	normal
CO2-48(C)	C4	normal
DEB125-1	C4	normal
CO2-124(C)	C4	del(7)q11.2q32
CO2-OS25	C2	normal
DEB135-10	135	normal
DEB135-24	135	normal
DEB135-S21	135	normal
DEB134-24	135	normal

B



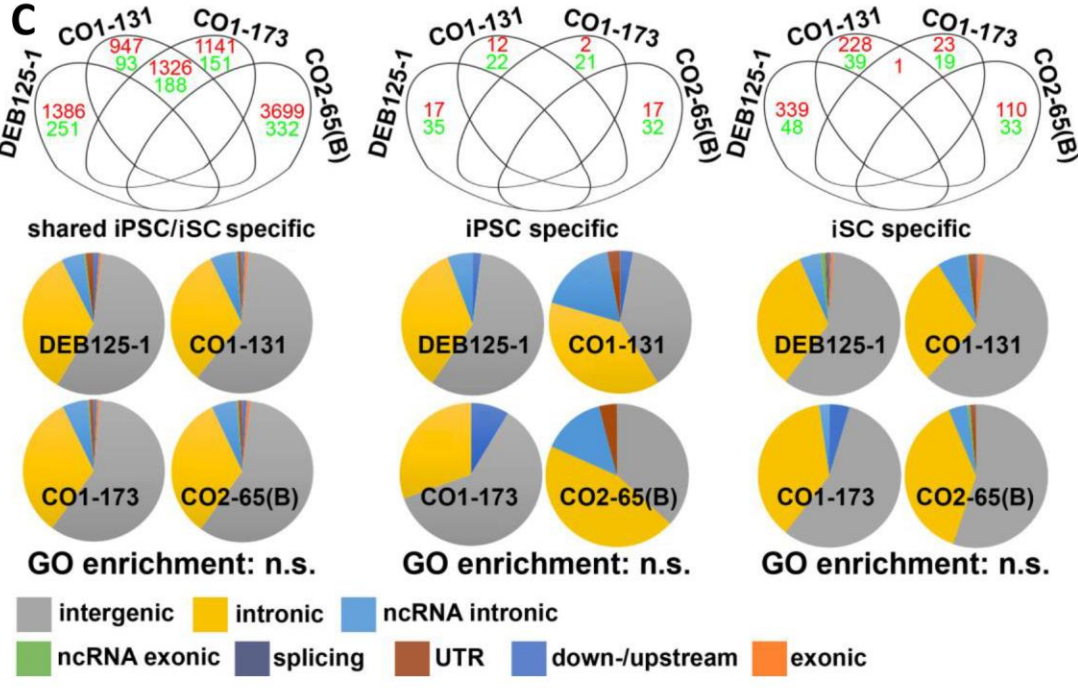
D



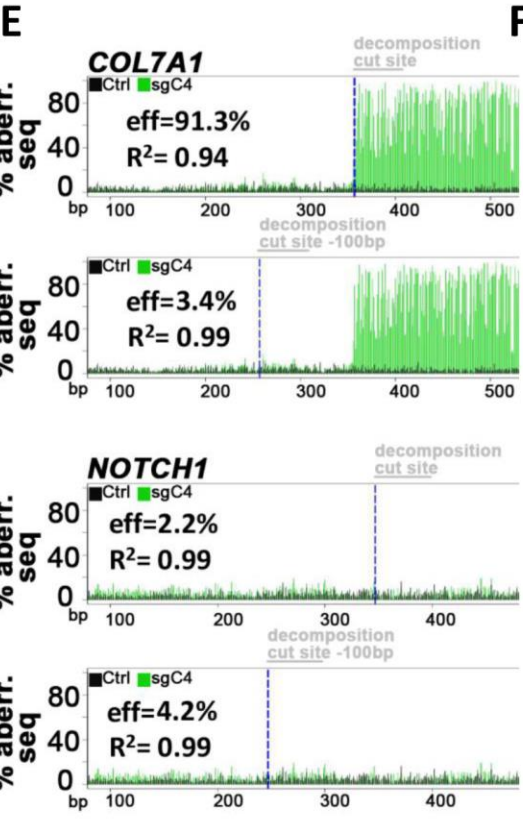
G

off-target	cutting	iPSC/iSC InDel
PAK4	No	No
HS3ST4	No	No
PAQR7/RP1-125I3.2	No	No
AC144450.2	3.2%	No
SLC25A29	2.1%	No
ZNF385A	No	No
CAPN12	No	No
NOTCH1	No	No
RP5-1159O4.1/MIOS	No	No
FXD1/CTD-2527I21.4	No	No
MARS	No	No
UBE2I	No	No
RP11-334A14.8/SLC1A7	0.3%	No
ZBED4	No	No
ZNF534	No	No
PADI3	No	No
VWA3A	No	No
UST	No	No
FAM207A	0.5%	No
PRR26	0.9%	No
PLXNA1	No	No
RBM10	No	No
VWF	No	No
OLFM1	0.2%	No
TIMM44	0.3%	No
LINC00710	No	No
OLAH	ND	No
INPP5A	ND	No
COL7A1 (on-target)	87.9%	CO1-131 / CO1-173 CO2-65(B)

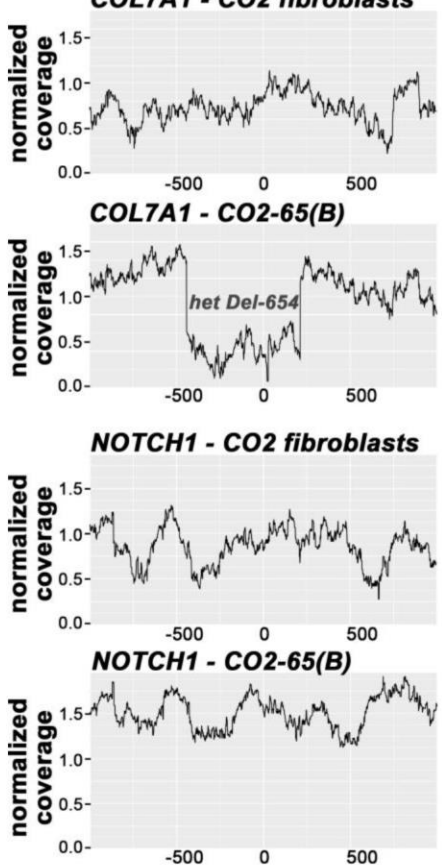
C



E



F



H

Patient fibroblasts	iPSC line	iSC
STAMP target (allele freq. %)	STAMP target (allele freq. %)	STAMP target (allele freq. %)
DEB125 CDKN1B (48.6%)	DEB125-1 CDKN1B (het. by sanger seq)	DEB125-1 CDKN1B (het. by sanger seq)
CO2 APC (47.2%) AR (3% by ddPCR)	CO2-65(B) APC (49.1%) AR (48.0%)	CO2-65(B) APC (48.4%) AR (57.9%)
	CO2-48(C) APC (49.2%) AR (47.7%)	CO2-48(C) APC (47.1%) AR (50.2%)
CO1 BRCA2 (48.3%)	CO1-131 BRCA2 (46.3%)	CO1-131 BRCA2 (48.9%)
	CO1-173 BRCA2 (48.3%)	CO1-173 BRCA2 (48.3%)

Figure 5

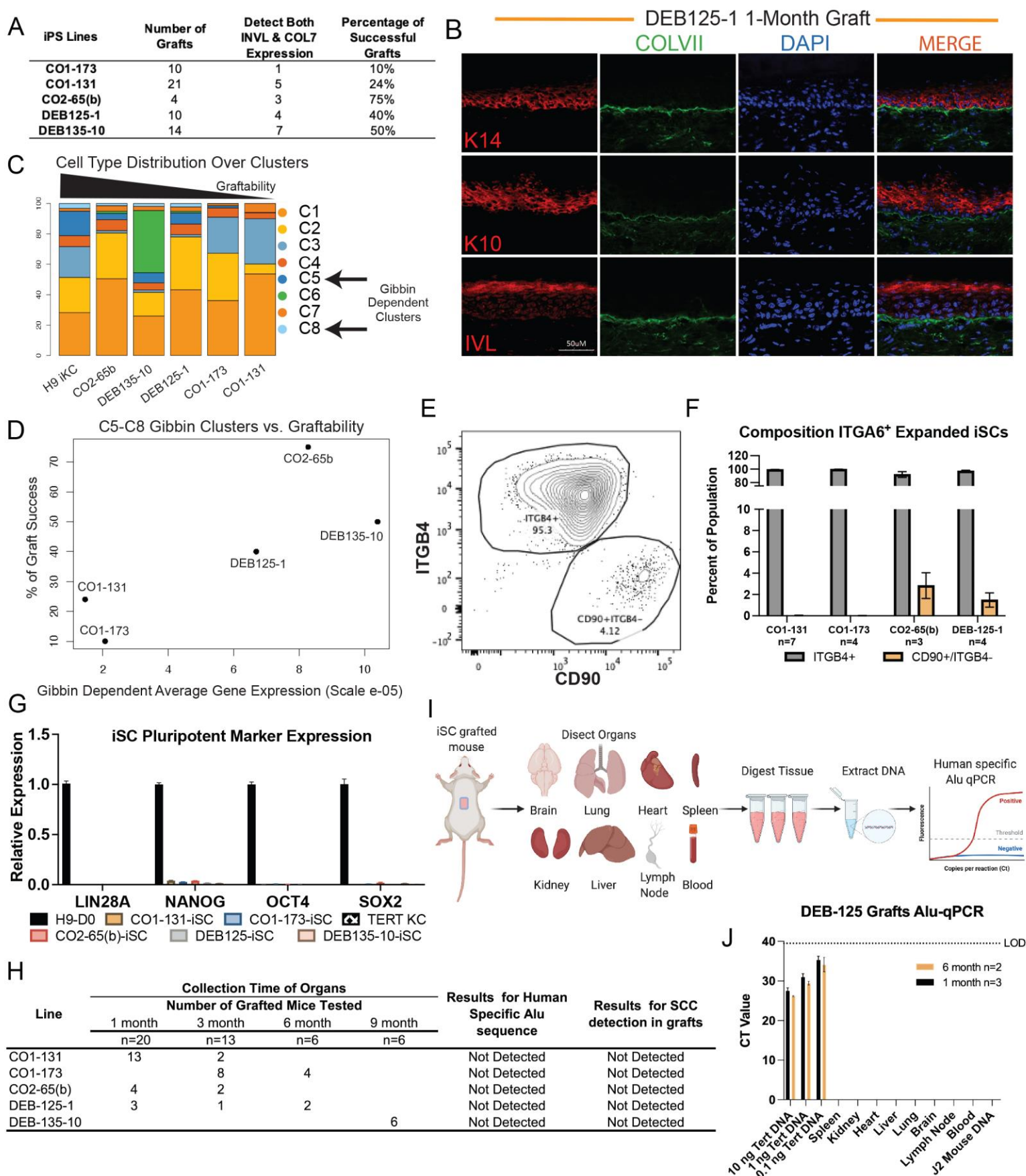


Fig S1

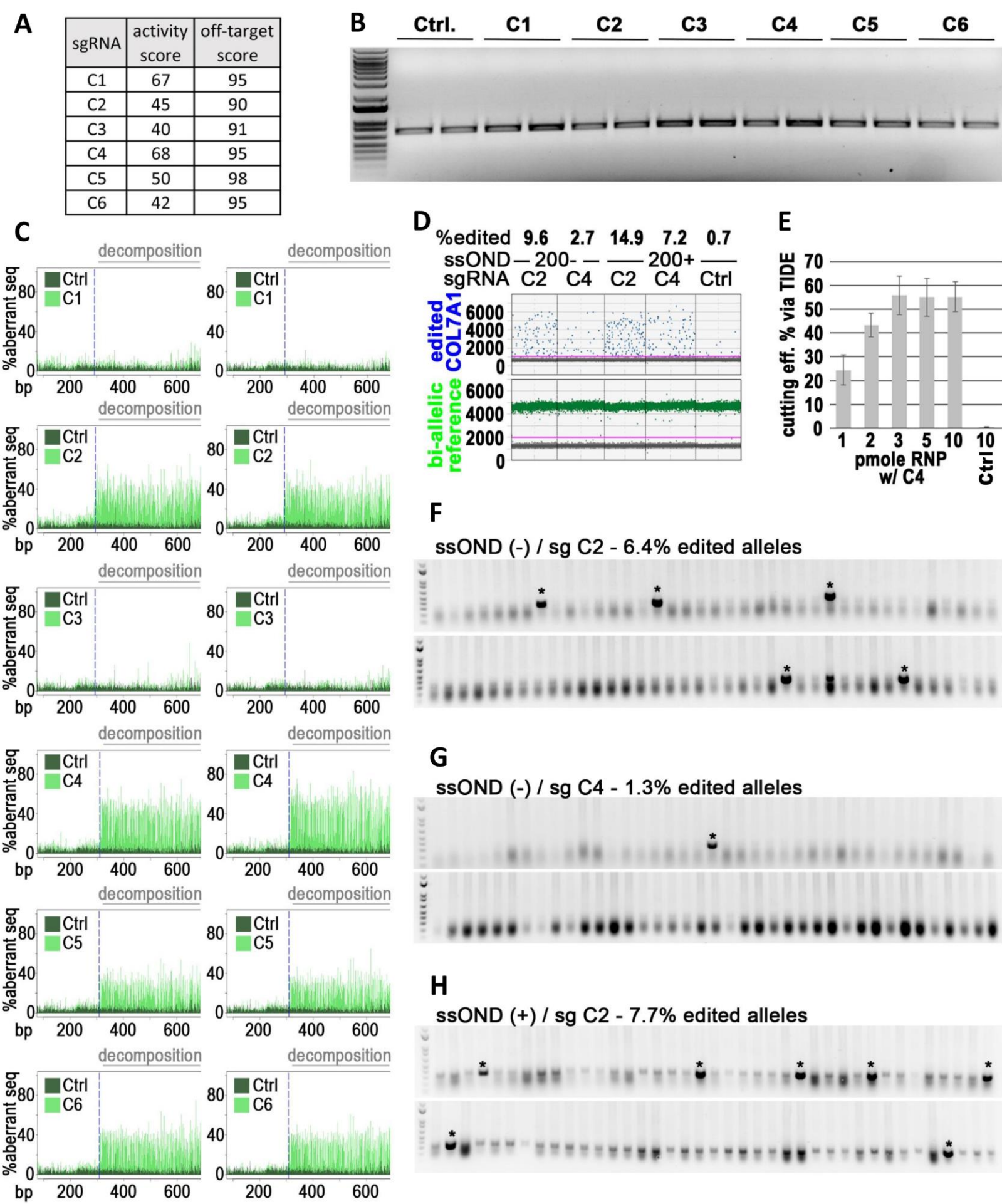
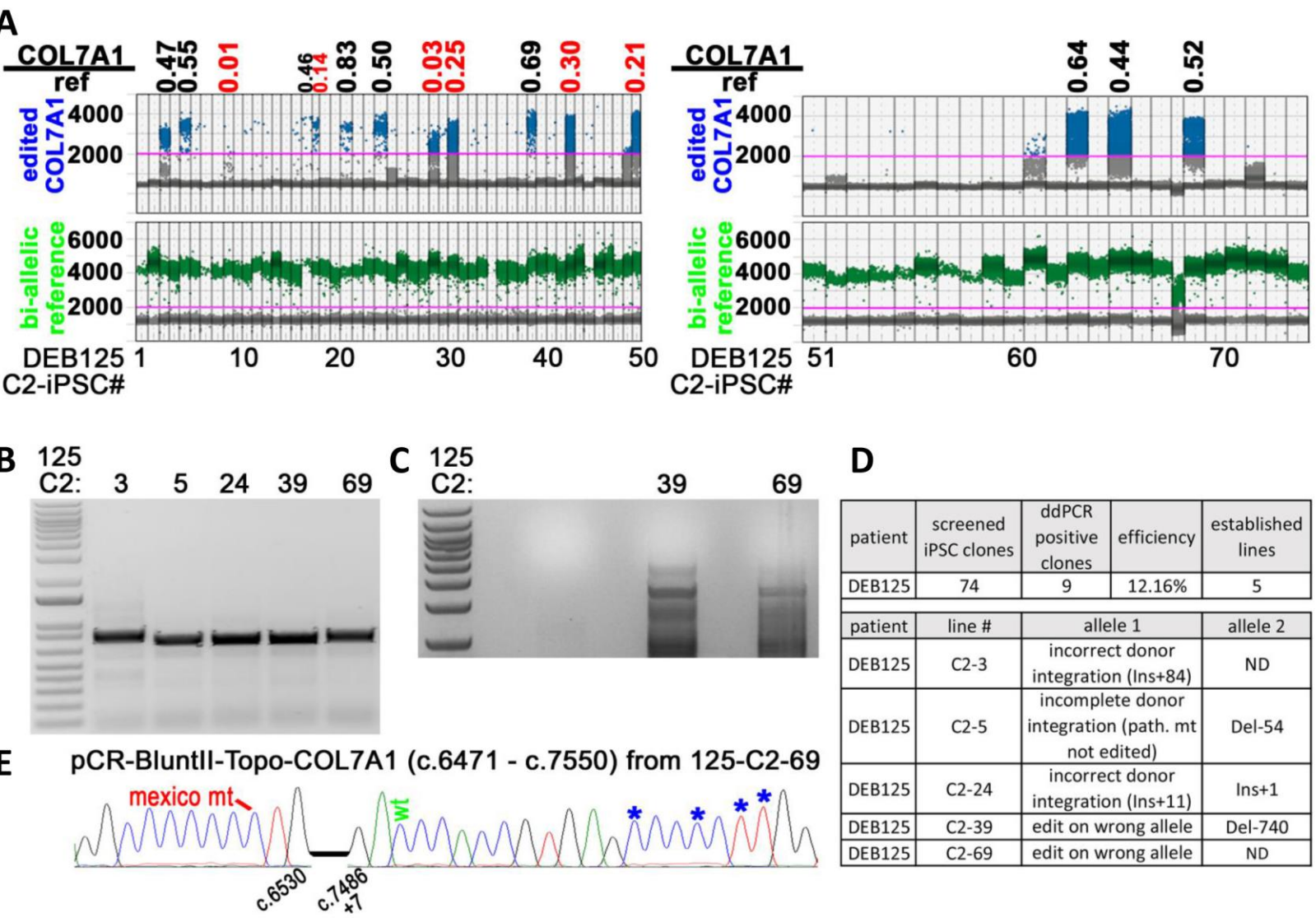
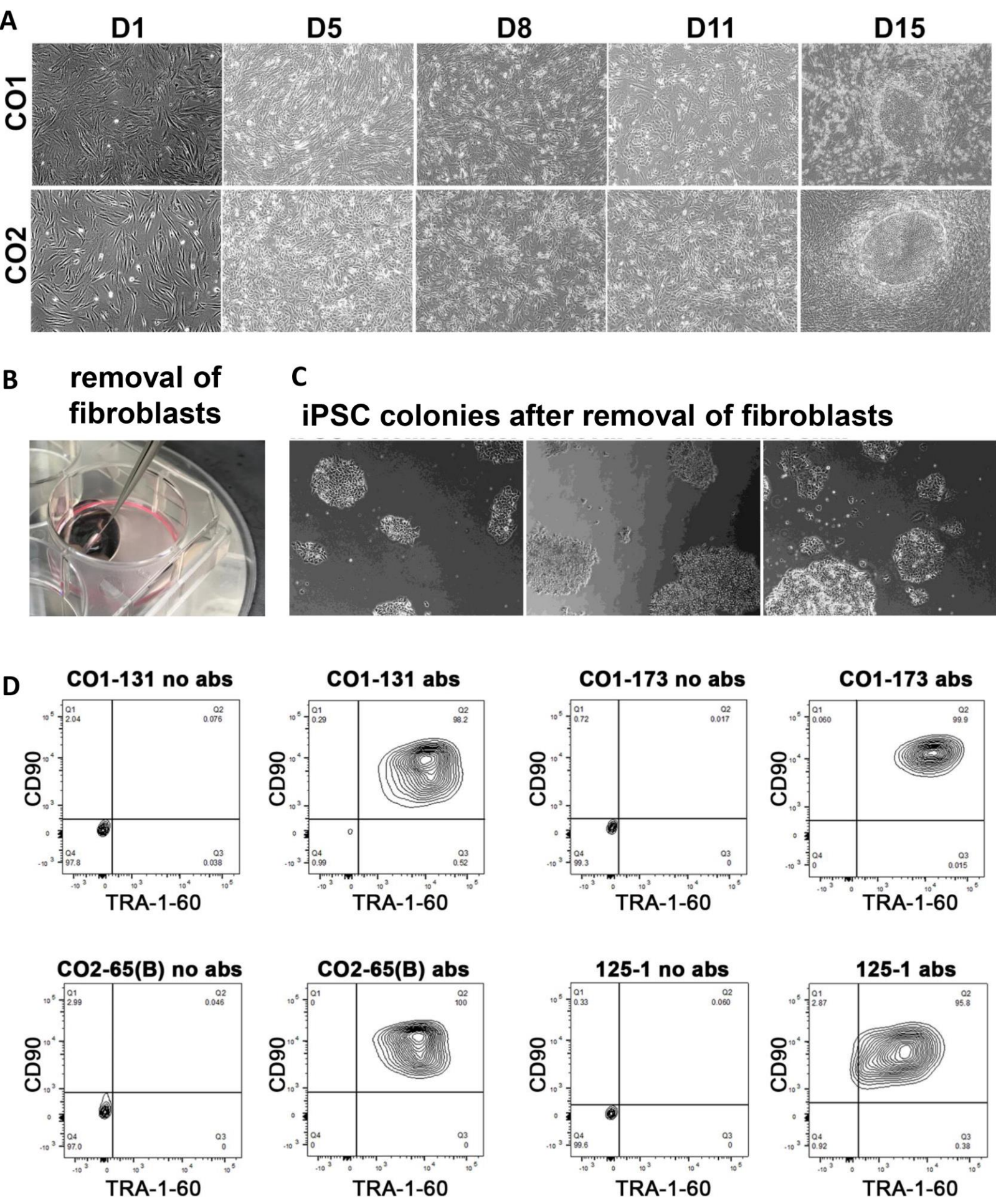
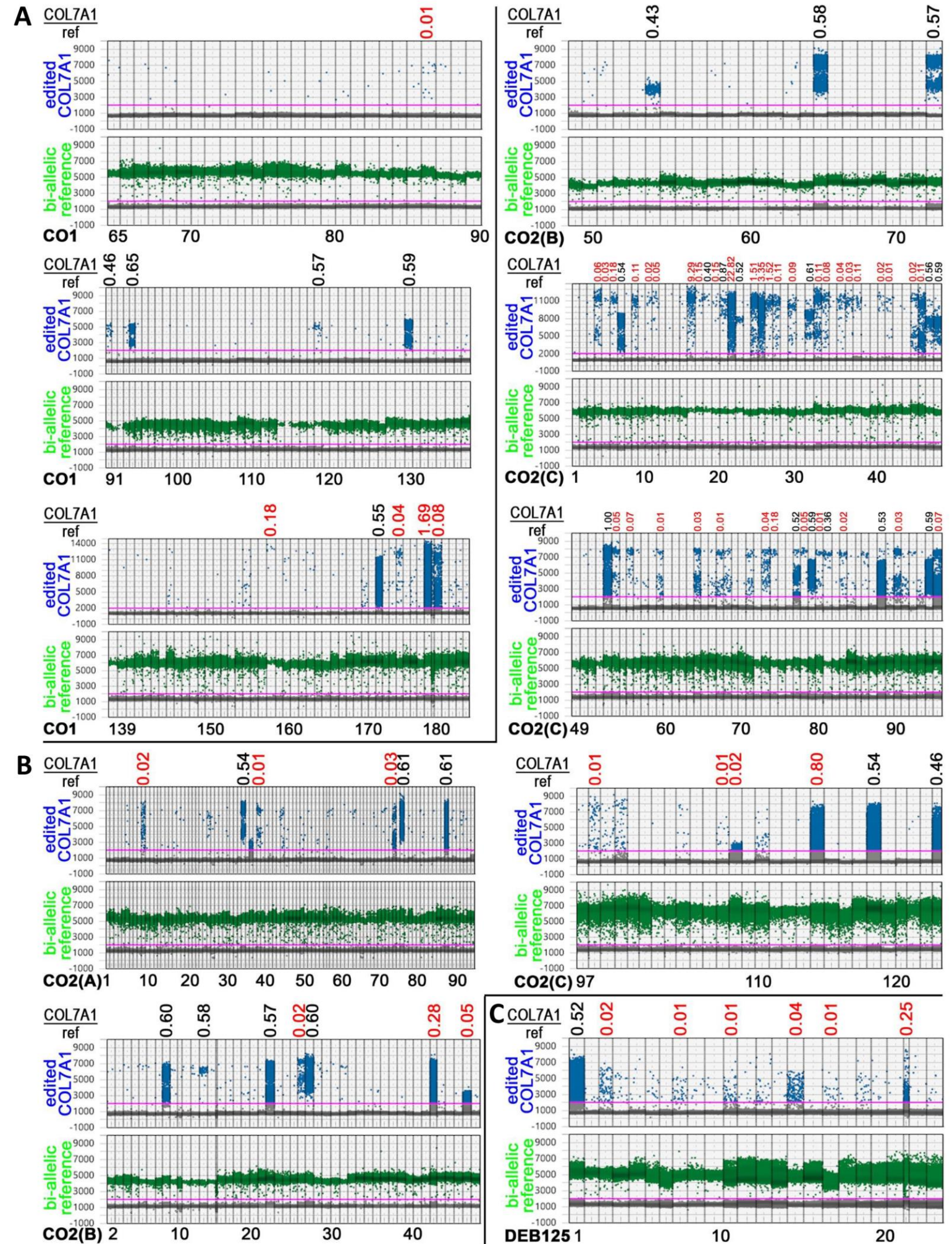
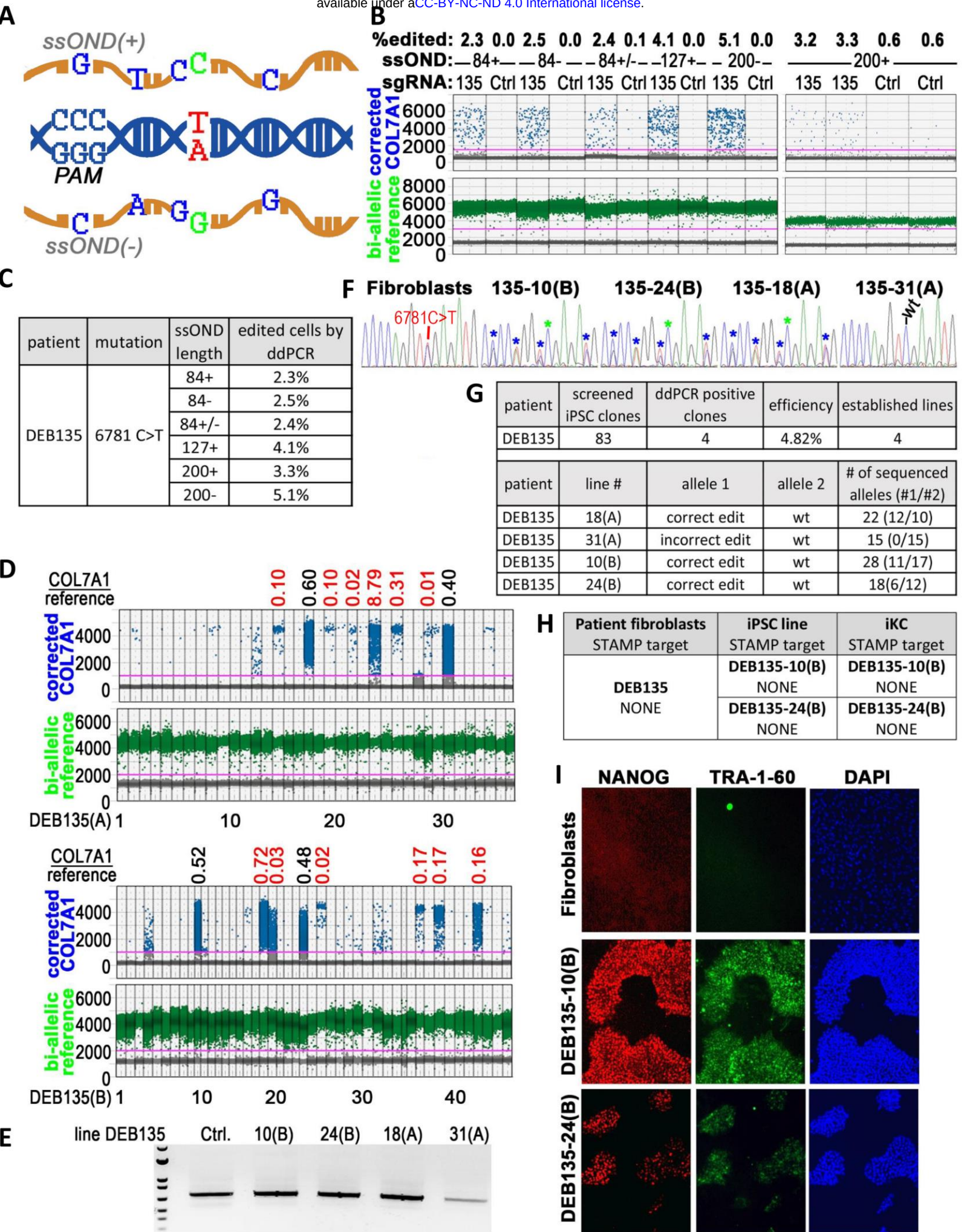


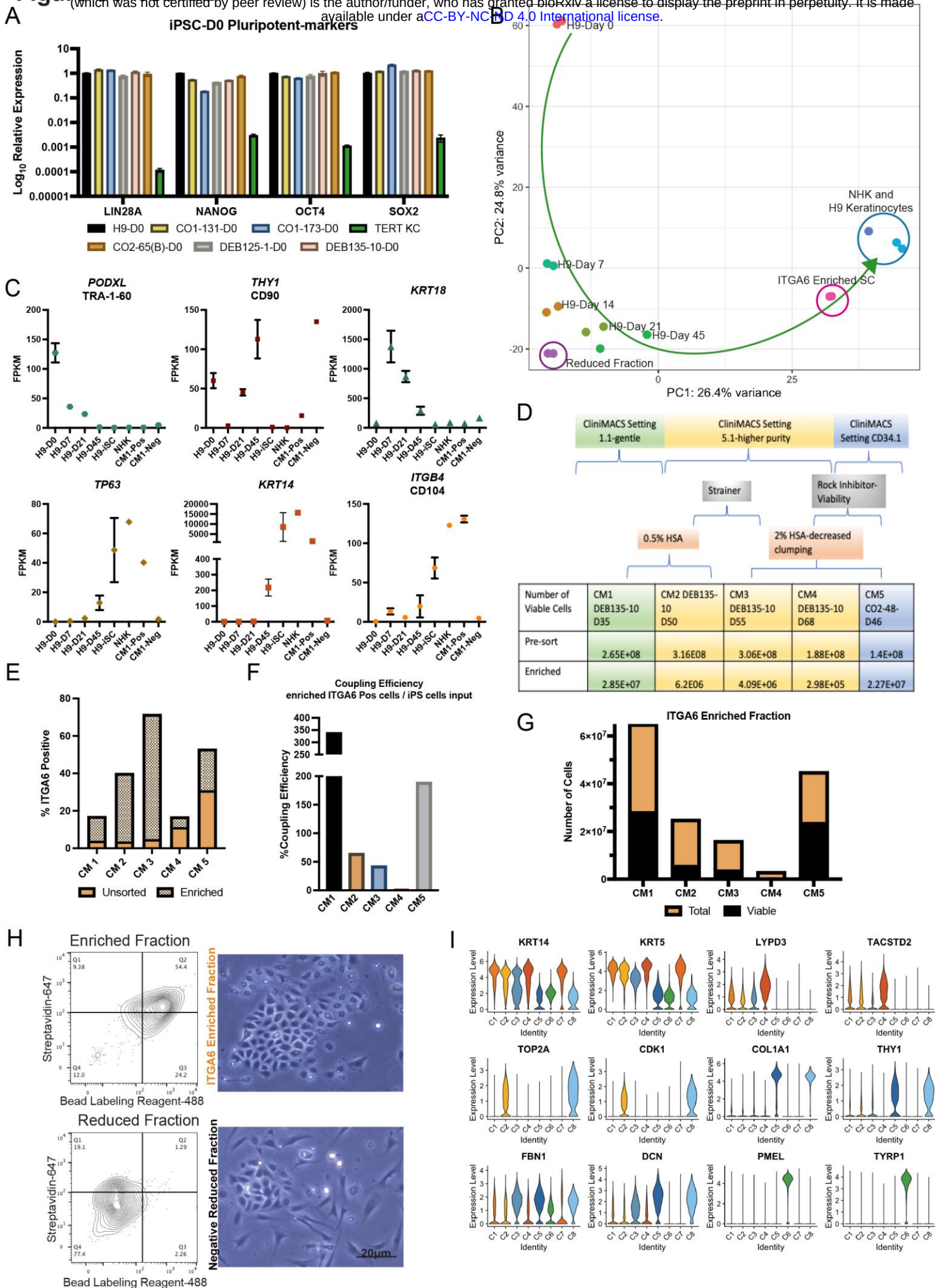
Fig S2

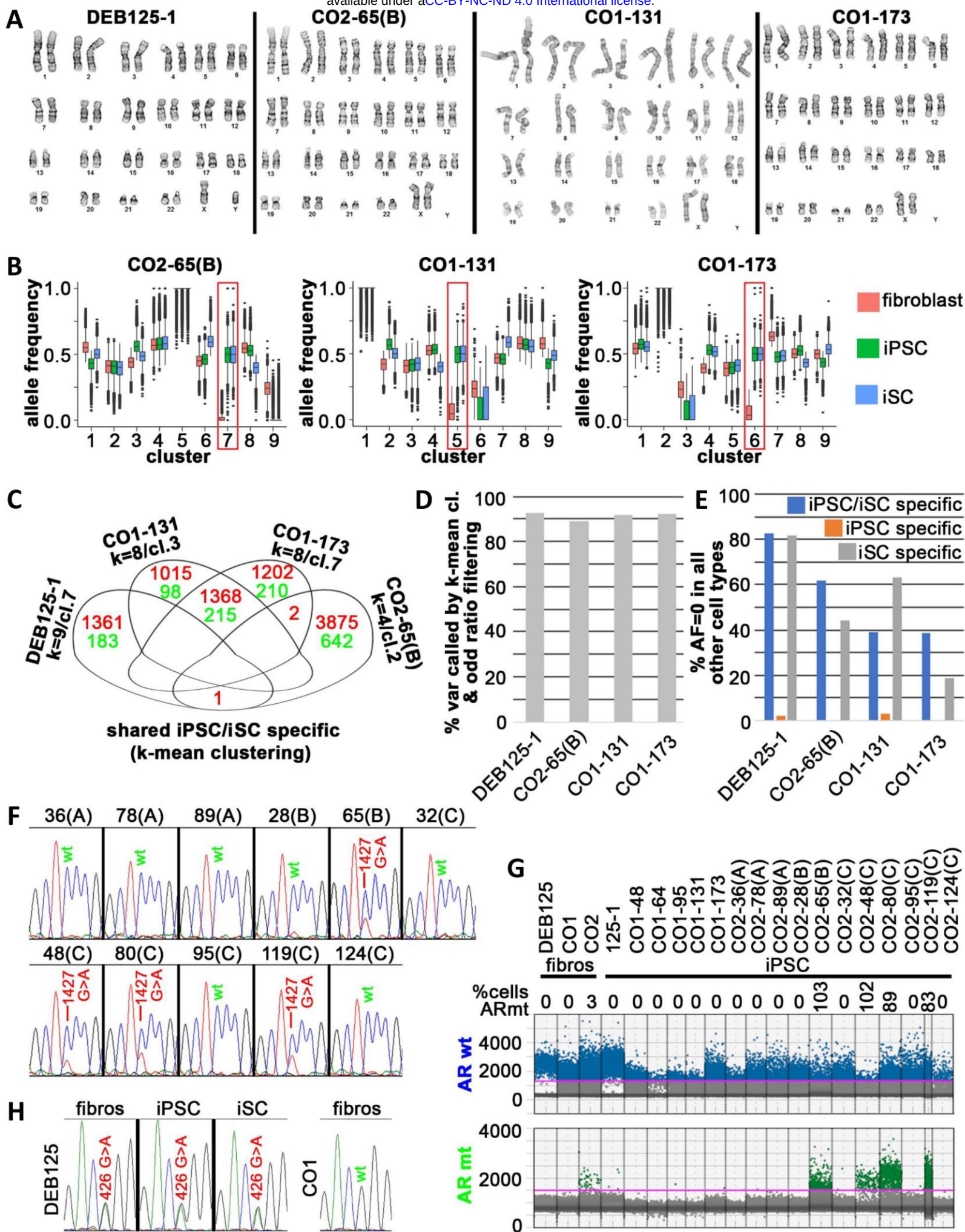


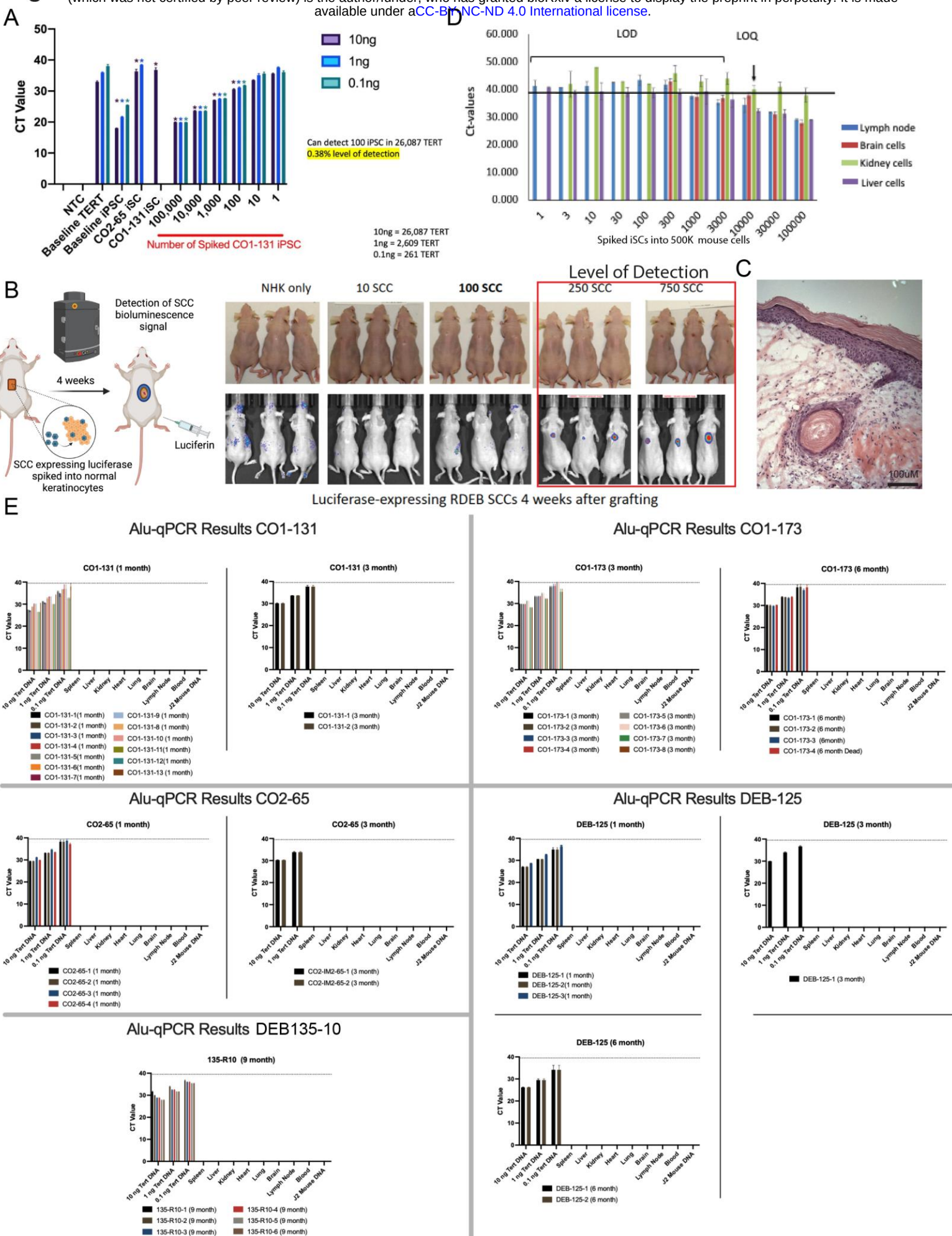












APPENDIX 2

Manuscript: Warshauer et al., 2021

ORIGINAL ARTICLE

Ancestral patterns of recessive dystrophic epidermolysis bullosa mutations in Hispanic populations suggest sephardic ancestry

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Abstract

Recessive dystrophic epidermolysis bullosa (RDEB) is a rare genodermatosis caused by mutations in the gene coding for type VII collagen (COL7A1). More than

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800 different pathogenic mutations in *COL7A1* have been described to date; however, the ancestral origins of many of these mutations have not been precisely identified. In this study, 32 RDEB patient samples from the Southwestern United States, Mexico, Chile, and Colombia carrying common mutations in the *COL7A1* gene were investigated to determine the origins of these mutations and the extent to which shared ancestry contributes to disease prevalence. The results demonstrate both shared European and American origins of RDEB mutations in distinct populations in the Americas and suggest the influence of Sephardic ancestry in at least some RDEB mutations of European origins. Knowledge of ancestry and relatedness among RDEB patient populations will be crucial for the development of future clinical trials and the advancement of novel therapeutics.

KEYWORDS

epidermolysis bullosa, genetics, genodermatoses

1 | INTRODUCTION

Recessive dystrophic epidermolysis bullosa (RDEB) is a rare genodermatosis characterized by severe skin fragility and blistering resulting in chronic wounds with progressive fibrosis (Mittapalli et al., 2019), caused by mutations in the gene coding for type VII collagen (*COL7A1*) (Hovnanian et al., 1994). More than 800 different pathogenic mutations in *COL7A1* have been described to date (Stenson et al., 2017); however, the ancestral origins of these mutations have not been precisely identified. Furthermore, little knowledge exists of the descendants of isolated Sephardic communities on the Iberian Peninsula, populations that characteristically propagate recessive diseases such as RDEB (Nogueiro et al., 2015a; Nogueiro et al., 2015b). The successful identification of common origins in distinct RDEB populations will provide an important resource as new treatments involving gene editing approaches for RDEB and other severe genetic skin diseases become viable for early clinical trials. In this study, 32 Hispanic RDEB patient samples carrying common *COL7A1* gene mutations from the Southwestern United States, Mexico, Chile, and Colombia were genotyped to investigate the ancestral origins for *COL7A1* mutations and determine the extent to which shared ancestry contributes to RDEB in these populations.

2 | RESULTS

The 32 Hispanic RDEB patients included in this study from the Southwestern United States, Mexico, Chile, and Colombia (see Table 1) carry pathogenic *COL7A1* gene mutations that were genotyped with different sequencing techniques and confirmed by Sanger Sequencing.

To further investigate ancestral patterns in the Hispanic RDEB population, a principal component analysis (PCA) and admixture analysis were performed (Alexander et al., 2009) on genomewide genotyping data alongside a reference panel consisting of

500 individuals from African, Middle Eastern, and European ancestry (Chang et al., 2015). In the PCA, the majority of the individuals are scattered between European and Native American populations (Figure 1). For Admixture, we identified three different European components, evident in Figures 2 and S1, modal in Western Europe (blue), Middle East and the Caucasus (red), and the Arabian peninsula (green), supporting the presence of Sephardic ancestry. For the American component, we identified at $K = 8$, a high variation in ancestral components, with an almost complete overlap between ancestry and population, possibly reflecting isolation and inbreeding dynamics, well-known in Jewish populations. Furthermore, Admixture analysis shows similar European and Native American ancestral components in the majority of the RDEB patients, with a minor, but consistent contribution from African groups (pink in Figure 2). A non-negative least squares (NNLS) haplotype-based method also supported overriding European and Native American origins, yet the majority (84%) of individuals harbor a small component ($>2\%$) of North African and Middle-Eastern ancestry (Figure 3, shown in green), which may result from interactions between North Africa or Jewish populations and Iberian populations. The evidence of North African and Middle-Eastern lineage substantiates, at least in part, the influence of Sephardic Jews in the Hispanic RDEB population.

To further explore the origin of these mutations, we estimated local ancestry across the *COL7A1* locus in all 32 RDEB patients using a reference panel consisting of African, European, and Native American populations (see Methods and Materials). Pathogenic mutations at the locus were determined separately from the local ancestry and are thus not phased with the local ancestry estimates, however most patients (25/32) had just one ancestry at both copies of the locus, allowing us to gain insights into the ancestral source of the 20 mutations present on these haplotypes (Table 2). Seventeen of the mutations appear in patients with a single ancestry at both copies of *COL7A1* (Table 2). Two additional mutations (c.7708delG and c.5532 + 1G > T) are present only in individuals with either a single ancestry at both copies of the locus or mixed ancestry at the locus, and may

TABLE 1 COL7A1 mutations in Hispanic RDEB patients

FTDNA kit no.	Mutation 1	Mutation 2	Sephardic	Test site
MK40784	c.6527_6528insC	c.8329C > T	21%	Chile
730,451	c.2470insG	c.2470insG	18%	Mexico
MK40790	c.6527_6528insC	c.5856 + 1G > T	15%	Chile
MK40779	c.7485 + 1G > A	c.4635 + 5G > A	10%	Colombia
MK42684	c.2470insG	c.2470insG	9%	Mexico
730,456	c.2470insG	c.2470insG	7%	Mexico
662,380	c.7485 + 5G > A	c.7485 + 5G > A	8%	Colorado
MK40771	c.8200G > A	c.4965C > T	7%	Colombia
MK40789	c.7708delG	c.7876-1G > A	5%	Chile
662,381	c.7485 + 5G > A	c.7485 + 5G > A	4%	Colorado
MK40776	c.7651G > C	c.4012G > A	4%	Colombia
MK40782	c.6781C > T	c.2044C > T	<2%	Colombia
MK40777	c.1584G > T	c.1584G > T	—	Colombia
MK40787	c.2005C > T	c.4342-2A > G	—	Chile
MK42706	c.5108G > A	c.IVS23-1G > A	—	Mexico
730,454	c.2470insG	c.2470insG	—	Mexico
MK40794	c.2992 + 2 T > G	c.6527_6528insC	—	Chile
MK40795	c.3264_5293del	c.5532 + 1G > T	—	Chile
MK40785	c.3759 + 2 T > G	c.3759 + 2 T > G	—	Chile
MK40772	c.425A > G	c.8833 T > C	—	Colombia
MK40775	c.4510G > T	c.4510G > T	—	Colombia
MK40778	c.4678G > A	c.4678G > A	—	Colombia
MK40793	c.5532 + 1G > T	c.8245G > A	—	Chile
MK40792	c.5932C > T	c.5932C > T	—	Chile
MK40780	c.6091G > A	c.6091G > A	—	Colombia
MK40783	c.6527_6528insC	c.6527_6528insC	—	Chile
MK40788	c.6527_6528insC	c.8528-1G > A	—	Chile
MK40773	c.6527_6528insC	c.5604 + 1G > A	—	Colombia
MK40791	c.7708delG	c.7708delG	—	Chile
MK40786	c.7708delG	c.8393 T > G	—	Chile
MK40774	c.8046 + 6G > A	c.5047C > T	—	Colombia
MK42683	c.8709del11	c.G2899del11	—	Mexico

thus be attributed to a single ancestral population in our data. Notably, a single mutation, c.2470insG, appears to have originated independently in both ancestral European and Native American populations. In total, 10 of the mutations appear to have arisen on Native American haplotypes, 8 on European haplotypes, and 4 are observed only in individuals with mixed ancestry and cannot be determined.

In order to evaluate the temporal origins of COL7A1 mutations in our dataset, we looked for shared haplotypes between patients and identified an enrichment of identity by descent (IBD) in the region surrounding the COL7A1 gene on chromosome 3 (Figure 4), supporting recent shared ancestry among at least some of the patients, even though none are known to be closely related. From among the 32 RDEB patients, we identified three different IBD clusters, each

representing a single shared haplotype, indicating recent shared ancestry between these individuals (Table 3).

The largest cluster is comprised of 4 individuals (2 from Chile, 1 from Colombia, and 1 from Mexico) (Table 3, Cluster A). The observation that $\frac{3}{4}$ of the patients carry at least one copy of the pathogenic RDEB mutation c.6527_6528insC mutation, with no other COL7A1 mutation being shared by all individuals in the cluster, suggests that the haplotype shared by individuals in the cluster carries the c.6527_6528insC mutation. It is possible that the pathogenic mutation of the patient who does not carry the c.6527_6528insC mutation was incompletely characterized, or that the shared haplotype does not encompass the mutation. Local ancestry indicates that the shared haplotype is of European origin.

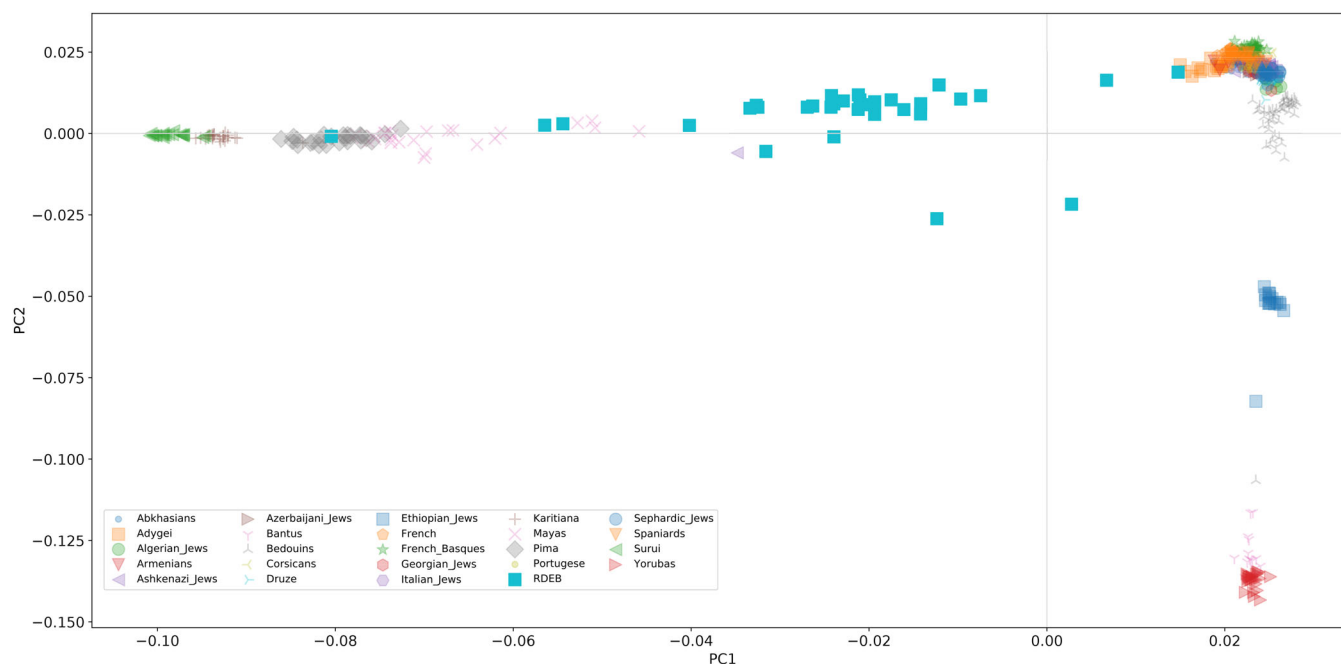


FIGURE 1 A Principal Component Analysis (PCA) was carried out on a dataset composed of 516 individuals in total, including 32 RDEB samples. The RDEB samples are marked with a teal blue square “■” and represent substantial admixture with Native American and European individuals, including Sephardic Jews represented with light blue circles in the right upper corner “●”. The legend in the left lower corner denotes the color markers exhibited in the PCA analysis. The X and Y axis refer to Principal Component (PC) 1 and 2, respectively

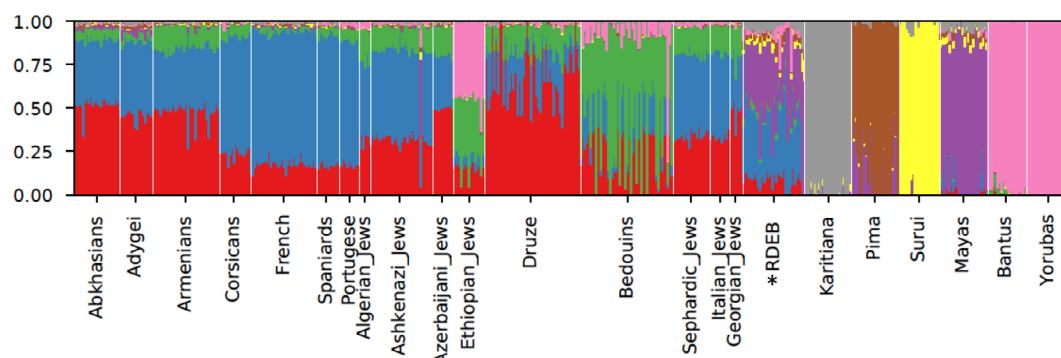


FIGURE 2 Admixture analysis. Population structure of 32 RDEB samples inferred by Admixture analysis. Each individual is represented by a vertical (100%) stacked column of genetic components proportions shown in color for K8 (K = number of subpopulations). The RDEB population is marked with an asterisk

A second IBD haplotype is shared by two RDEB patients from Mexico (Table 3, Cluster B). Both patients are homozygous for the c.2470insG mutation, and both are homozygous for the shared haplotype. Local ancestry across the *COL7A1* locus on the shared haplotype indicates that it is likely of European origin.

The third IBD haplotype is shared by two RDEB patients from Chile carrying the c.7708delG mutation (Table 3, Cluster C), one of whom is homozygous for the shared haplotype and the mutation. Native American local ancestry across the *COL7A1* locus in all patients suggests that this mutation arose in the Americas.

Multiple other mutations are observed multiple times in the RDEB patients without being part of an IBD cluster. We do not rule

out the possibility that at least some of these mutations may have been inherited from a common ancestor, but recombination in intervening generations has interrupted the inherited haplotype to a size below detection thresholds. On the other hand, it is also reasonable to consider that at least some of these mutations may have arisen independently.

3 | DISCUSSION

Admixed populations present challenges in understanding the history of disease, particularly in regions where each population can have a

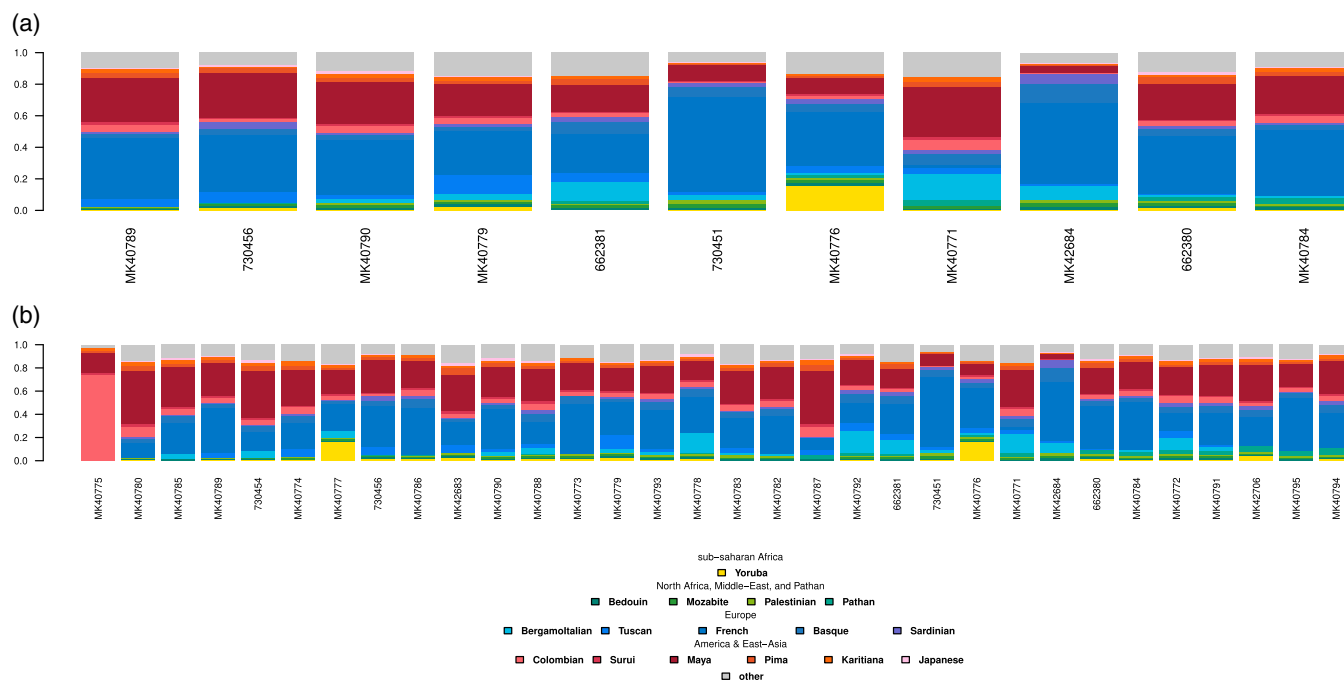


FIGURE 3 NNLS haplotype method. The NNLS Haplotype method exhibits a bar plot of the ancestral composition for a set of surrogate groups, as demonstrated in the legend. Implemented in CP and merged with HGDP sequences, each individual (recipient) was painted as a combination of genomic fragments inherited by all the HGDP panel (donors). Thirty-two Hispanic RDEB patient samples carrying common mutations in the *COL7A1* gene sorted according to Middle Eastern ancestry from the Southwestern United States, Mexico, Chile, and Colombia (a). Asterisks in (a) highlight the samples shown in (b). The analysis of RDEB samples with affinity to Sephardic origins sorted according to Middle Eastern ancestry demonstrate North African and Middle Eastern origins, highlighted in green, effectively supporting hidden genetic ancestry in heavily admixed individuals (b)

different admixture history. The high frequency of shared haplotypes harboring pathogenic RDEB mutations among unrelated patients in our data suggests that founder or other demographic events may have increased the prevalence of these pathogenic haplotypes in Hispanic populations in the Americas. We also present evidence that RDEB mutations in multiple Hispanic populations have ancestral origins from both European and Native American ancestral populations, with at least one mutation (c.2470insG) having origins from both ancestral populations. We note that because the number of observations of each mutation in our dataset is limited by our patient sampling, it is possible that other mutations presented here also have other origins that simply are not sampled in our patients. Thus, we cannot exclude the possibility that these other mutations have independent origins in other ancestral populations.

Eleven of the RDEB patients presented here had substantial amounts (>4%) of Sephardic ancestry using FamilyTreeDNA ancestry testing, a finding confirmed by a significantly higher proportion of European and American ancestry in these patients compared to the remainder of the RDEB patients (Figure 5). The finding is not entirely unexpected as it is known that Sephardic individuals arrived in the Americas in the late 15th century, and Sephardic ancestry has been previously detected in some Hispanic populations, with some Sephardic mutations being observed in former Spanish colonies such as the Southwestern US, Mexico, and El Salvador (Ellis et al., 1998; Ostrer, 2016; Shahrabani-Gargir

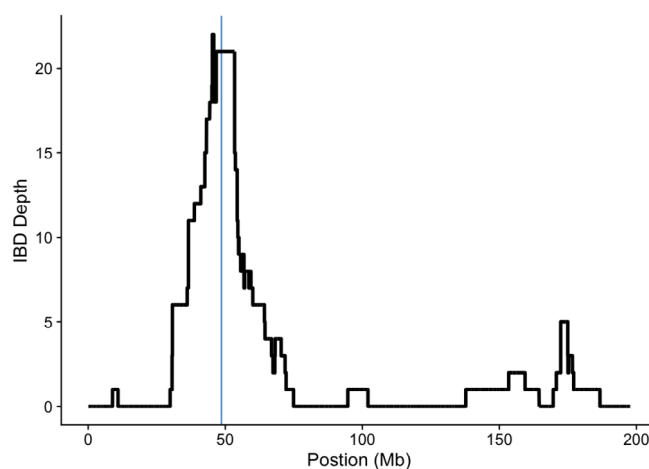


FIGURE 4 Identity by descent (IBD). Cumulative depth of identical by descent (IBD) segments from all pairwise sample comparisons across chromosome 3 (blue vertical line indicates the location of *COL7A1*)

et al., 1998; Struwing et al., 1995; Velez et al., 2012). Notably, significant admixture also occurred between North African non-Jewish populations and Sephardic Jews following their expulsion from Spain during the Inquisition (Aksentijevich et al., 1993; Campbell et al., 2012), which may explain the ancestral

TABLE 2 Counts of local ancestry at COL7A in RDEB patients for each mutation

Mutation	Counts of local ancestry at COL7A in RDEB patients for each mutation		
	AMR	EUR	Mixed
c.7485 + 5G > A	4	0	0
c.7708delG	3	0	1
c.2470insG	2	6	0
c.4510G > T	2	0	0
c.1584G > T	2	0	0
c.6091G > A	2	0	0
c.5108G > A	1	0	0
c.8200G > A	1	0	0
c.2005C > T	1	0	0
c.IVS23-1G > A	1	0	0
c.4965C > T	1	0	0
c.6527_6528insC	0	6	1
c.4678G > A	0	2	0
c.3759 + 2 T > G	0	2	0
c.5932C >	0	2	0
c.7651G > C	0	1	0
c.2992 + 2 T > G	0	1	0
c.3264_5293del	0	1	0
c.5532 + 1G > T	0	1	1
c.8709del11	0	1	0
c.425A > G	0	0	1
c.8046 + 6G > A	0	0	1
c.7485 + 1G > A	0	0	1
c.6781C > T	0	0	1

components in the NNLS related to African and Middle Eastern populations (Figures 2 and 3).

The recognition that the European haplotype shared by individuals carrying the c.6527_6528insC mutation represents patients from three separate populations could suggest that the arrival of the mutation on the haplotype predates European settlement. This knowledge would be consistent with estimates of the first occurrence of the c.6527_6528insC mutation more than 3000 years ago when pre-Roman communities settled in the Iberian Peninsula (Sanchez-Jimeno et al., 2013). Interestingly, this region was also home to a closed endogamous community of Sephardic Jews during a time period more than a millennium ago, coinciding with the estimated origin of the c.6527_6528insC founder mutation (Adams et al., 2008). The c.6527_6528insC mutation remains frequent on the Iberian Peninsula of Spain and it is possible that at least some Hispanic populations inherited this mutation through Hispanic or Sephardic migration. Data from the original source of Sephardic Jews on the Iberian Peninsula is limited and the question of Sephardic origins is challenging as Sephardic and Spanish ancestry signals are likely to be largely overlapping due to centuries of cohabitation (Álvarez-Álvarez et al., 2018; Nogueiro et al., 2015b).

From the analyses presented here, the observation that at least some RDEB mutations appear to have European origins lends

some support to the hypothesis that some of the pathogenic RDEB mutations presented here are of Sephardic origin. Population frequencies represented in gnomAD indicate slight enrichment with European populations in eight Hispanic RDEB mutations (Table S1), providing rationale for further investigation of Sephardic ancestry in these variants (Collins et al., 2020; Karczewski et al., 2020; Landrum et al., 2020). Interestingly, there are a few rare variants demonstrating enrichment with African populations, which may represent the Sephardic populations of North Africa (Gonçalves et al., 2005). Establishing more confident and specific inferences of the ancestral origins of the mutations present in the Hispanic RDEB patients will benefit from additional sampling from ancient and modern-day Sephardic populations as well as from the populations from which the RDEB samples were collected.

Gene-editing treatments for RDEB patients using guide RNAs specific to pathogenic mutations are anticipated in the near future (Bonafont et al., 2019; Mencía et al., 2018). As regulatory agencies may consider each guide RNA as a separate drug, separate clinical trials for each RNA would be required. Patient populations who share the same founder mutations therefore represent an increasingly important resource to facilitate early clinical trials and advance novel treatments.

TABLE 3 Counts of RDEB mutations and local ancestry populations within identity by descent COL7A1 haplotypes

IBD cluster ID	Count of patients in cluster	Count of patient haplotype zygosity for cluster (#homozygous/#heterozygous)	Count of patient mutation Zygosity (#homozygous/#heterozygous)										Local ancestry counts at COL7A1 in cluster		
			c.6527_6528insC	c.5604 + 1G > A	c.8528-1G > A	c.8709del11	c. G2899del11	c.2470insG	c.7708delG	c.7876-1G > A	AMR	EUR	Mixed		
A	4	0/4	1/2	0/1	0/1	0/1	0/1	0/1	0/0	0/0	0/0	0/0	0	3	1
B	2	2/0	0/0	0/0	0/0	0/0	0/0	0/0	2/0	0/0	0/0	0/0	0	2	0
C	2	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/1	2	0	0

4 | MATERIALS AND METHODS

Thirty-two RDEB homozygous and compound heterozygous patient samples from the Southwestern United States, Mexico, Chile, and Colombia with common mutations in COL7A1 included in this study were previously identified using different sequencing technologies and subsequently confirmed by Sanger sequencing (Table 1). Informed written consent was obtained from all patients in concordance with Institutional Review Board approval from the USA: Colorado Multiple Institutional Review Board (COMIRB no: 09-0192), Mexico: Universidad de Monterrey (132012-CE), Chile: Comité Ético Científico, Facultad de Medicina, Clínica Alemana—Universidad del Desarrollo (Project number 2013-145), and Colombia: Universidad del Rosario (CIE-UR DVO005 1149-CV1192).

4.1 | Illumina bead chip array and FTDNA family finder analysis

DNA samples were genotyped by GeneByGene, Inc. utilizing the Illumina Human OmniExpress BeadChip array and analyzed with the FTDNA Family Finder autosomal DNA test. The Family Finder test returns results for about 690,000 pairs of single nucleotide polymorphisms (SNPs) on the 22 pairs of autosomal chromosomes. Autosomal SNPs are clustered into sets about 50–100 SNPs long that are predefined based on the reliability, variability, average centimorgans (cM), and density of the SNPs. The Family Finder software then evaluates SNP sets for matching as half identical or a non-match based on an autosomal DNA algorithm demonstrating shared DNA segments. Adjacent SNP sets are also analyzed to see if they qualify as identical by descent (IBD) segments. A segment is considered a candidate autosomal match if it contains at least 500 SNPs, and it is at least 1 cM long. Then, proprietary rules based on total shared cM and longest segment cM are applied to infer whether the match is valid.

The RDEB DNA samples were then subset to approximately 245,000 unlinked SNPs and run through ADMIXTURE using FamilyTreeDNA's proprietary global reference panel called myOrigins2. The 24 global population clusters represent modern human genetic variation and results reflect admixture between historical gene pools. They include Sephardic, Ashkenazi, North and Central America, South America, British Isles, Scandinavia, Finland, West and Central Europe, Southeast Europe, East Europe, Iberia, West Middle East, East Middle East, Asia Minor, North Africa, East Central Africa, South Central Africa, West Africa, Central Asia, South Central Asia, Siberia, Northeast Asia, Southeast Asia, and Oceania. Affinity to Sephardic origins is determined significant by at least a 4% match to the Sephardic cluster (Chacón-Duque et al., 2018).

4.2 | Identity by descent analysis

We merged the genotyping data from RDEB patients with genotypes from a combined reference dataset consisting of individuals from the

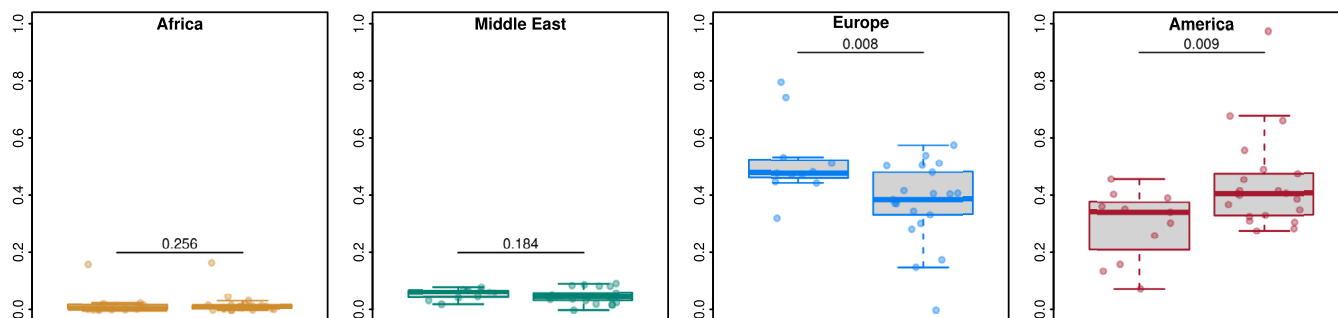


FIGURE 5 Ancestries proportions. This figure represents the ancestry proportions for Africa, the Middle East, Europe and America. Within each panel, the left hand side bar graph represents RDEB samples with Sephardic ancestry and the right hand side bar graph represents RDEB samples without Sephardic ancestry. The 11 RDEB samples with Sephardic ancestry clearly demonstrate significantly greater European ancestry and less American ancestry than RDEB samples without Sephardic ancestry. There are no significant differences noted among the African or Middle Eastern ancestry

Human Genome Diversity Project (HGDP) (Cann et al., 2002) and 1000 Genomes (Genomes Project et al., 2010; Genomes Project et al., 2012; Genomes Project et al., 2015), and phased the entire merged dataset using EAGLE2 (Loh et al., 2016) with default parameters. Phased haplotypes were then fed through iLASH to detect identical by descent (IBD) regions greater than or equal to 3 cM between pairs of RDEB patients. Cumulative IBD depth at each marker was calculated using a custom python script that counts the total number of times each locus is part of an IBD segment shared between two individuals. We used DASH (Gusev et al., 2011) with a minimum cluster size of 3 and a minimum haplotype length of 3 cM to identify clusters of patients that share the same IBD haplotypes across the entirety of COL7A1.

4.3 | Local ancestry analysis

We used phased haplotypes (see “Identity by Descent Analysis”) from RDEB patients in RFMix (Maples et al., 2013) (version 1.5.4) with a reference panel consisting of European and nonadmixed African populations (ESN, GWD, MSL, LWK, YRI) from 1000 Genomes and 107 indigenous American individuals from HGDP to train the random forests. The estimates for local ancestry presented in the article are generated from the RFMix Viterbi output.

4.4 | PCA and admixture analysis

In order to explore the ancestry of the analyzed individuals, an explorative analysis was carried out using a PCA and Admixture approach (Alexander et al., 2009). We merged the genotype data with publicly available genome-wide datasets (Behar et al., 2010; Behar et al., 2013; Kushniarevich et al., 2015; Ongaro et al., 2019; Tambets et al., 2018; Tamm et al., 2019; Yunusbayev et al., 2012; Yunusbayev et al., 2015) using PLINK 1.9, a widely used program for research in population genetics (Chang et al., 2015). After merging, SNPs and individuals characterized by less than 3% and 5% of missing data were

retained for a total of 87,181 SNPs, and 516 individuals. A PCA was carried out using the PCA flag in PLINK1.9 (Chang et al., 2015).

The Admixture analysis was carried out on the dataset with random seed and cross-validation based on data resampling. Although the most supported configuration is $K = 9$, we are showing $K = 8$, for which European and the Middle East ancestries show a reasonable degree of differentiation (Figure S1).

4.5 | CP and NNLS

The ancestry composition of the analyzed individuals was explored utilizing haplotype method implemented in ChromoPainter (CP, Lawson et al., 2012). The analyzed samples were merged with the HGDP sequences in order to increase the density of the data (Bergström et al., 2019). First, the sequence data SNPs coordinates were lifted from Ghch38 to hg19 using Picard Tools (Picard, 2020). The merged dataset was subsequently phased using shapeit ver 2. using default settings (Delaneau et al., 2011). Second, each individual (recipient) was painted as a combination of genomic fragments inherited by all the HGDP panel (donors), an effective way to unlock the hidden genetic ancestry in heavily admixed individuals (Montinaro et al., 2015; Ongaro et al., 2019). The nuisance parameters were set as $M = 0.0018$ and $n = 409$, estimated based on expectation maximization run in a subset of five individuals from each population.

The resulting copying vectors were subsequently normalized and each RDEB patient individual ancestry was reconstructed as a combination of copying vectors from all the populations in the HGDP dataset, using a modified version of the NNLS function of R software implemented in GlobeTrotter (Hellenthal et al., 2014; Leslie et al., 2015). The results for the RDEB patients are summarized in Figure 4. Populations contributing on average less than 1% were labeled as others.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Emily Mira Warshauer, Adam Brown, Ignacia Fuentes, Karl Skorecki, Eli Sprecher, Dennis R. Roop. Formal Analysis: Emily Mira Warshauer, Adam Brown, Ignacia Fuentes, Jonathan Shortt, Chris Gignoux, Francesco Montinaro, Mait Metspalu, Leila Youssefian, Hassan Vahidnezhad, Joanna Jacków, Angela M. Christiano, Jouni Uitto, Óscar R. Fajardo-Ramírez, Julio C. Salas-Alanis, John A. McGrath, Liliana Consuegra, Carolina Rivera, Paul A. Maier, Goran Runfeldt, Doron M. Behar, Karl Skorecki, Eli Sprecher, Francis Palisson, David A. Norris, Adam Brown, Igor Kogut, Ganna Bilousova, Dennis R. Roop. Writing—original draft: Emily Mira Warshauer, Ignacia Fuentes, Jonathan Shortt, Chris Gignoux, Francesco Montinaro, Dennis R. Roop. Writing—review and editing: Emily Mira Warshauer, Adam Brown, Ignacia Fuentes, Jonathan Shortt, Chris Gignoux, Francesco Montinaro, Paul A. Maier, Goran Runfeldt, Karl Skorecki, Dennis R. Roop.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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APPENDIX 3

Manuscript: Pavlova et al., 2024

The Development of an Advanced Model for Multilayer Human Skin Reconstruction In Vivo

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Abstract

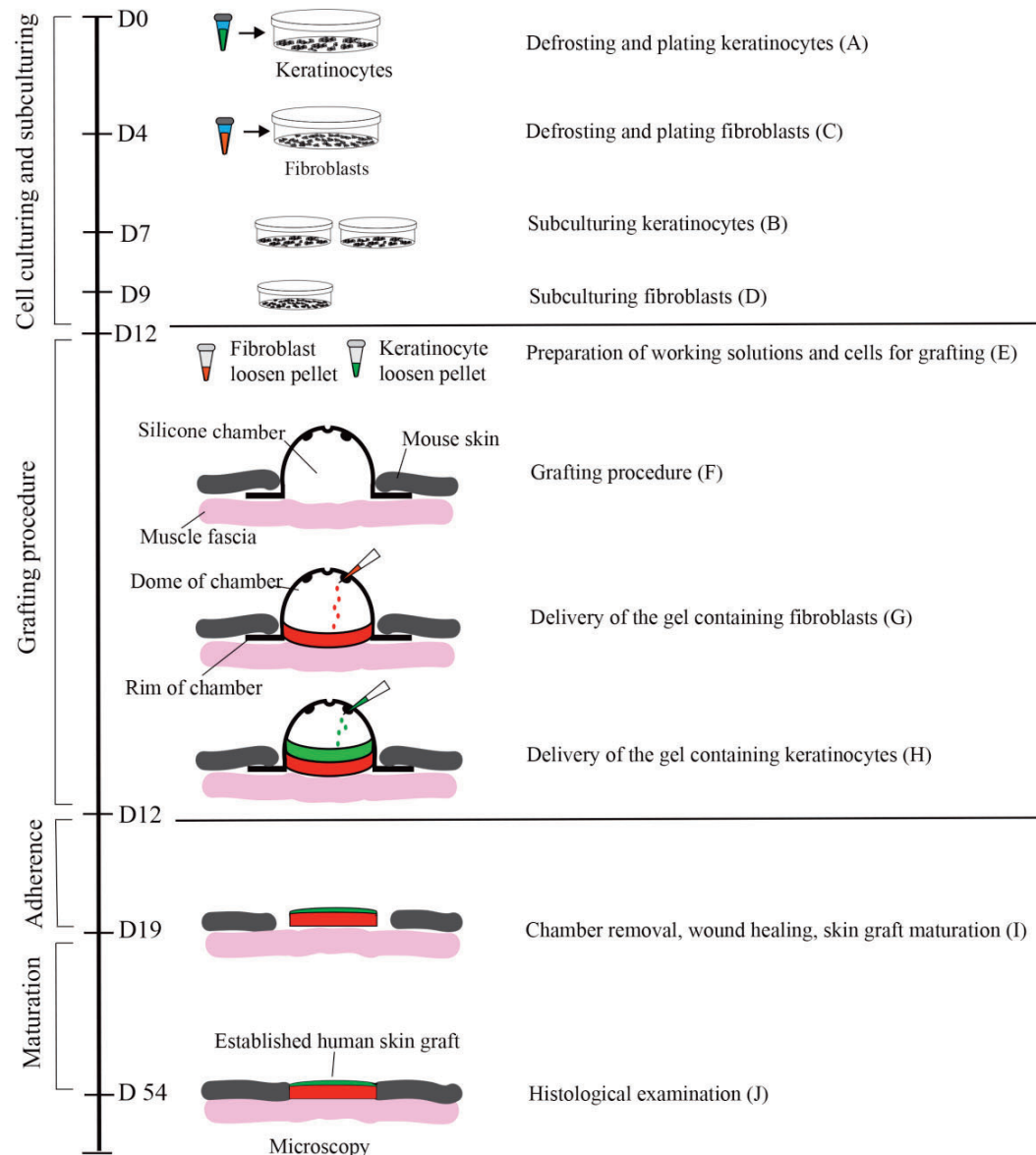
Human skin reconstruction on immune-deficient mice has become indispensable for in vivo studies performed in basic research and translational laboratories. Further advancements in making sustainable, prolonged skin equivalents to study new therapeutic interventions rely on reproducible models utilizing patient-derived cells and natural three-dimensional culture conditions mimicking the structure of living skin. Here, we present a novel step-by-step protocol for grafting human skin cells onto immunocompromised mice that requires low starting cell numbers, which is essential when primary patient cells are limited for modeling skin conditions. The core elements of our method are the sequential transplantation of fibroblasts followed by keratinocytes seeded into a fibrin-based hydrogel in a silicone chamber. We optimized the fibrin gel formulation, timing for gel polymerization in vivo, cell culture conditions, and seeding density to make a robust and efficient grafting protocol. Using this approach, we can successfully engraft as few as 1.0×10^6 fresh and 2.0×10^6 frozen-then-thawed keratinocytes per 1.5 cm^2 of the wound area. Additionally, it was concluded that a successful layer-by-layer engraftment of skin cells in vivo could be obtained without labor-intensive and costly methodologies such as bioprinting or engineering complex skin equivalents.

Key features

- Expands upon the conventional skin chamber assay method (Wang et al., 2000) to generate high-quality skin grafts using a minimal number of cultured skin cells.
- The proposed approach allows the use of frozen-then-thawed keratinocytes and fibroblasts in surgical procedures.
- This system holds promise for evaluating the functionality of skin cells derived from induced pluripotent stem cells and replicating various skin phenotypes.
- The entire process, from thawing skin cells to establishing the graft, requires 54 days.

Keywords: Human skin equivalent, Fibrin-based hydrogel, Multilayered skin graft, In vivo skin model, Regenerative medicine

Graphical overview



Generation of a human skin equivalent on an immunodeficient mouse using a fibrin-based grafting system.

A schematic of the protocol is shown. Cultured keratinocytes and fibroblasts resuspended in a fibrin-based gel are delivered as layers into a silicon chamber inserted underneath the skin of an immunocompromised mouse. First, a fibrin gel containing encapsulated fibroblasts (up to 2×10^6 per 1.4 cm^2 wound) is delivered into the chamber and allowed to solidify for 15 minutes. Second, a fibrin gel containing $1.0\text{--}2.0 \times 10^6$ keratinocytes is applied on top of the fibroblast layer. On day 7 post-grafting, the chamber is removed, and the wound with the graft is allowed to heal for 4–5 weeks. During healing, a scab forms and eventually falls off. By day 54, the graft is fully established.

Background

Progress in mouse genetics has provided a powerful research tool for elucidating the mechanisms of epidermal stem cell commitment and skin homeostasis. However, given the dramatic difference in skin structure between humans and mice, one must be cautious in applying the insights gained in studying mouse skin to human skin, especially when investigating skin diseases. For example, the inbred genetic background of mice can influence the phenotype resulting from disease-associated mutations. Humans are of mixed genetic background, and this complexity results in phenotypical variations and a different penetrance of genetically defined diseases, such as epidermolysis bullosa, a group of rare inherited skin blistering diseases. For this reason, developing complex in vivo human xenograft models is critical to advance our understanding of human skin conditions and developing new therapeutic interventions.

Several human xenograft models have been previously published, including a skin flap assay (Qiao et al., 2008), human skin transplantation-like approaches for grafting dermal-epidermal equivalents onto opened wounds of recipient mice (Escamez et al., 2004; Martinez-Santamaria et al., 2012; Yanez et al., 2015; Jorgensen et al., 2020), and chamber grafting assay (Wang et al., 2000; Diette et al., 2020). While promising, all these assays suffer from several limitations. They include (1) the complexity of the procedure that requires the generation of 3D skin equivalents in cell culture conditions before the transplantation onto a mouse, and (2) the limited availability and proliferative capacity of primary human cells, especially cells derived from patients with rare inherited skin diseases. A chamber grafting assay (Wang et al., 2000; Diette et al., 2020) provides a simplified procedure for grafting primary human skin cells since the assay promotes the self-assembly of fibroblasts and keratinocytes in an in vivo environment without the need to produce skin equivalents in a dish. However, successful production of human skin equivalents in the grafting assay still requires at least 5×10^6 primary keratinocytes per 1.4 cm^2 of the wound area, which is a significant number when the availability of primary patient cells is limited. In addition, since this assay relies on the ability of keratinocytes and fibroblasts to self-assemble and form the dermis and epidermis, this limits the ability to incorporate other cell types that normally reside in the skin into an appropriate skin layer. Therefore, we aimed to modify the grafting chamber assay by decreasing the number of primary skin cells necessary for transplantation and mimicking the natural three-dimensional structure of the human skin in vivo to make a more viable in vivo model to study human skin conditions.

To achieve this, we modified the delivery of human keratinocytes and fibroblasts into the grafting chamber by first suspending these cells in a fibrin-based hydrogel. We then performed the sequential transplantation of fibroblasts followed by keratinocytes to recapitulate two major layers of the skin, the dermis and the epidermis, in a controlled manner. Due to its simplicity, this method can be easily optimized by incorporating additional cell types of different origins and ensuring that they are targeted explicitly into the dermis or the epidermis. In addition to grafting primary keratinocytes and fibroblasts, this system can be appropriate for testing the functionality of skin cells derived from induced pluripotent stem cells. The system is also applicable for testing novel skin transplantation systems for potential clinical applications, including cell harvesting and application techniques for treating cutaneous skin defects and pigmentation disorders.

Materials and reagents

Biological materials

1. Nude mice as recipients for grafting: homozygous nude Foxn1^{nu} , formerly Hfh11^{nu} , females, 6–8 weeks (Jackson Laboratory, Nu/J, #002019; <https://www.jax.org/strain/002019>). Alternatively, the homozygous NOD-Prkdc^{scid} mice can be recipients for grafting (Jackson Laboratory, NOD.Cg-Prkdc^{scid}/J, # 001303). Both nude and NOD-SCID mouse strains show efficient engraftment of human cells. However, NOD-SCID mice require depilation on the back before surgery and often before graft harvest. Therefore, careful consideration should be given to selecting the appropriate mouse strain for immunological studies to ensure compatibility with the experimental requirements and to minimize any potential confounding factors related to hair removal (Waldron-Lynch et al., 2012; Cristobal et al., 2021).

Note: Before preparing cell cultures, ensure that all mice are healthy and fully acclimated to the facility for at least a week. Ensure that the weight of the mice is above 21 g.

2. Fibroblasts, primary, neonatal (ATCC, catalog number: PCS-201-010)
3. Human epidermal keratinocytes, primary, neonatal (HEKn) (ThermoFisher, catalog number: C0015)

Reagents

1. DMEM/F12 (1:1) (ThermoFisher, catalog number 11320-033)
2. Pooled human AB serum derived (Innovative Research, ISERAB)
3. MEM-NEAA (ThermoFisher, catalog number: 11140050)
4. GlutaMax (ThermoFisher, catalog number: 35050061)
5. 2-Mercaptoethanol (ThermoFisher, catalog number: 21985023)
6. L-Ascorbic acid (Sigma-Aldrich, catalog number: A4544)
7. Hydrocortisone (Sigma-Aldrich, catalog number: H0888)
8. Antibiotic/Antimycotic (ThermoFisher, catalog number: 15240062)
9. Human bFGF (ThermoFisher, catalog number: PHG0263)
10. Human EGF (ThermoFisher, catalog number: PHG0313)
11. EpiLife medium (ThermoFisher, catalog number: MEPI500CA)
12. EpiLife defined growth supplement (EDGS) (ThermoFisher, catalog number: S0125)
13. Bovine collagen solution, type I, 3 mg/mL (Advanced BioMatrix, catalog number 5005).
14. Fetal bovine serum, qualified, one shot, raw (ThermoFisher, catalog number: A31605-01)
15. Aprotinin (Sigma-Aldrich, catalog number: A6279-10ML)
16. Fibrinogen from human plasma (Sigma-Aldrich, catalog number: F3879-1G)
17. Thrombin from human plasma (Sigma-Aldrich, catalog number: T4393-100UN)
18. DPBS without calcium and magnesium (ThermoFisher, catalog number: 14190-144)
19. 0.25% Trypsin-EDTA (ThermoFisher, catalog number: 25200-056)
20. Accutase (StemCell, catalog number: 7920)
21. CRYO defined, animal component free freezing medium, 2× (ZenBio, catalog number: CNT-CRYO-50)
22. CryoStor CS10, 1× (StemCell, catalog number: 100-1061)
23. Anti-mouse keratin (K)1 antibody, Rabbit, 1:500 dilution (BioLegend, catalog number: 905602)
24. Anti-mouse/human K14 antibody, Chicken, 1:2,000 dilution (BioLegend, catalog number: 906004)
25. Anti-human Loricrin antibody, Rabbit, 1:2,000 dilution (Abcam, catalog number: 176322)
26. Anti-human Vimentin antibody, Mouse, 1:500 dilution (Abcam, catalog number: 16700)
27. Anti-Rabbit-488 secondary antibody (Invitrogen, catalog number: A11008)
28. Anti-Chicken-594 secondary antibody (Invitrogen, catalog number: A11042)
29. Anti Mouse-594 secondary antibody (Invitrogen, catalog number: 11032)
30. Formalin solution, neutral buffered, 10% (Sigma-Aldrich, catalog number: HT501128)

Solutions

Stock solutions

1. Thrombin stock solution (25 U/mL): made from powder (see Recipes)
2. Fibrinogen stock solution (40 mg/mL): made from powder (see Recipes)
3. Bovine collagen solution, type I (3 mg/mL): ready to use

Working solutions

1. Complete FEM medium (fibroblasts culturing) (see Recipes)
2. Complete EpiLife medium (keratinocytes culturing) (see Recipes)
3. Collagen solution (coating for keratinocyte culture) (see Recipes)
4. Fibrin gel containing fibroblasts (see Recipes)
5. Fibrin gel containing keratinocytes (see Recipes)

Recipes

Recipes for stock solutions

1. Thrombin stock solution

Working under aseptic conditions in a biosafety cabinet, reconstitute thrombin in sterile DPBS (without Ca^{2+} and Mg^{2+}) at a concentration of 25 U/mL. Make an aliquot and freeze at $-20\text{ }^{\circ}\text{C}$. The day before the surgery, defrost the thrombin solution overnight at $2\text{--}8\text{ }^{\circ}\text{C}$. Keep thrombin on ice before applying it for hydrogel preparation in the grafting procedure. The solution should be used promptly; however, it can be refrigerated at $2\text{--}8\text{ }^{\circ}\text{C}$ for up to three days.

2. Fibrinogen stock solution

Working under aseptic conditions in a biosafety cabinet, reconstitute fibrinogen in sterile DPBS (without Ca^{2+} and Mg^{2+}) at a concentration of 40 mg/mL stepwise. First, lay a thin layer of fibrinogen powder on top of warm ($37\text{ }^{\circ}\text{C}$) DPBS and let it soak for 5 min at $37\text{ }^{\circ}\text{C}$; repeat the soaking procedure with the rest of the fibrinogen. Second, put the tube with the fibrinogen-saline solution in a water bath at $37\text{ }^{\circ}\text{C}$ for 30–60 min and agitate the tube gently every 10 min. Ensure that there are no clumps of dry fibrinogen on the tube walls. Third, leave the tube on the shaker at $2\text{--}8\text{ }^{\circ}\text{C}$ overnight to complete the preparation of the fibrinogen stock solution. If a significant number of undissolved clumps are left, repeat the 30-min incubation at $37\text{ }^{\circ}\text{C}$. Do not exceed 1 h of incubation at $37\text{ }^{\circ}\text{C}$. The fibrinogen-saline solution **must not** be vortexed. Fibrinogen should be filter-sterilized using a $0.22\text{ }\mu\text{m}$ syringe filter. Do not use vacuum filtration since this will lead to the breakdown of the molecule during the filtration. Make 50–100 μL aliquots and freeze at $-20\text{ }^{\circ}\text{C}$.

Note: Alternatively, bovine fibrinogen and thrombin can be used.

Recipes for working solutions

1. Complete FEM medium (Table 1)

Prepare FEM medium by supplementing DMEM/F12 with 5% human serum, $0.5\times$ MEM-NEAA, $0.5\times$ GlutaMAX™ supplement, 55 μM Gibco™ 2-mercaptoethanol, $1\times$ HyClone Antibiotic/Antimycotic, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 1 $\mu\text{g}/\text{mL}$ hydrocortisone. Perform $0.22\text{ }\mu\text{m}$ sterile filtration and store at $4\text{ }^{\circ}\text{C}$ for up to one month. Before application, add human bFGF to a final concentration of 12 ng/mL and human EGF to a final concentration of 5 ng/mL.

Table 1. Composition of complete FEM medium

Reagent	Stock concentration	Final concentration	Amount
DMEM/F12 (1:1)			92.763 mL
Human serum	100%	5%	5 mL
MEM-NEAA	100 $\times$	0.5 $\times$	0.5 mL
GlutaMax Supplement	100 $\times$	0.5 $\times$	0.5 mL
2-Mercaptoethanol	55 mM	55 μM	0.1 mL
Ascorbic acid	50 mg/mL	50 $\mu\text{g}/\text{mL}$	0.1 mL
Hydrocortisone	5 mg/mL	1 $\mu\text{g}/\text{mL}$	0.02 mL
Antibiotic/Antimycotic	100 $\times$	1 $\times$	1 mL
1. Sterilize using a $0.22\text{ }\mu\text{m}$ filter; 2. Add bFGF and EGF as shown below AFTER filtration			
Reagent	Stock concentration	Final concentration	Amount
bFGF	100 $\mu\text{g}/\text{mL}$	12 ng/mL	0.012 mL
EGF	100 $\mu\text{g}/\text{mL}$	5 ng/mL	0.005 mL
Total	n/a	n/a	0.005 mL

2. Complete EpiLife medium (Table 2)

Prepare complete EpiLife medium by adding EpiLife Defined Growth Supplement (EDGS) following the manufacturer's instruction. Add antibiotic/antimycotic.

Table 2. Composition of complete EpiLife Medium

Reagent	Stock concentration	Final concentration	Amount
EpiLife basal medium	n/a	n/a	500 mL
EDGS	100×	1×	5 mL
Antibiotic/Antimycotic	100×	1×	5 mL
Total	n/a	n/a	510 mL

3. Collagen solution

To prepare the collagen working solution for the coating of the tissue culture dishes, dilute the 3 mg/mL bovine collagen stock solution to a final working concentration of 30 µg/mL in 1× DPBS. Use fresh.

4. Fibrin gel containing fibroblasts (Table 3)

To prepare the fibrin gel containing fibroblasts, first make working Solution 1. To make working Solution 1, supplement DMEM/F12 1:1 with 1× Antibiotic/Antimycotic, 1% raw FBS, and aprotinin. Mix components in an Eppendorf tube and keep the tube on ice until ready to graft. Just before grafting, mix Solution 1 with fibroblasts and then add fibrinogen and thrombin. Mix components and use for grafting.

Critical: Do not add fibroblasts, fibrinogen, and thrombin into Solution 1 until ready to graft*.

Table 3. Composition of the gel containing fibroblasts

Reagent	Stock concentration	Final concentration	Amount
Solution 1			
DMEM/F12 (1:1)	n/a	n/a	291.7 µL
Antibiotic/Antimycotic	100×	1×	4 µL
Raw FBS	100%	1%	4.1 µL
Aprotinin	(3-7 TIU/mg protein)	n/a	23.5 µL

Just before grafting, add fibroblasts, fibrinogen, and thrombin as shown below.

Reagent	Stock concentration	Final concentration	Amount
Fibroblasts*	n/a	2×10^6	50 µL
Fibrinogen*	40 mg/mL	1.73 mg/mL	17.3 µL
Thrombin*	25 U/mL	0.59 U/mL	9.4 µL
Total	n/a	n/a	400 µL

5. Fibrin gel containing keratinocytes (Table 4)

To prepare the fibrin gel containing keratinocytes, first make working Solution 2. To make a working Solution 2, supplement EpiLife medium with 1× Antibiotic/Antimycotic and aprotinin. Mix components in an Eppendorf tube and keep the tube on ice until ready to graft. Just before grafting, mix Solution 2 with keratinocytes and then add fibrinogen and thrombin. Mix components and use for grafting.

Critical: Do not add keratinocytes, fibrinogen, and thrombin into Solution 2 until ready to graft**.

Table 4. Composition of the gel containing keratinocytes

Reagent	Stock concentration	Final concentration	Amount
Solution 2			
EpiLife	n/a	n/a	295.8 µL
Antibiotic/Antimycotic	100×	1×	4 µL
Aprotinin	(3-7 TIU/mg protein)	n/a	23.5 µL

Just before grafting, add keratinocytes, fibrinogen, and thrombin as shown below.

Reagent	Stock concentration	Final concentration	Amount
Keratinocytes**	n/a	2×10^6	50 μ L
Fibrinogen**	40 mg/mL	1.73 mg/mL	17.3 μ L
Thrombin**	25 U/mL	0.59 U/mL	9.4 μ L
Total	n/a	n/a	400 μ L

Note: For a large-scale experiment, the working hydrogel solutions for the dermal and epidermal layers can be prepared as a combined mix to facilitate the generation of multiple grafts simultaneously. To maximize efficiency, 2–4 grafts can be performed in a single experimental setup. Optional: recalculate the volumes for Solution 1 and Solution 2 based on the number of grafts.

Equipment

1. Silicone chambers [produced by Qure Medical, 1810 Renaissance Boulevard, Sturtevant, WI, 53177, (262) 417-1307, <http://www.quire-med.com>, according to the following dimensions: 12 mm inner diameter (ID), 20 mm outer diameter (OD), 10 mm tall (Part# 12–24, upper chamber)]. Chambers are made of Momentive LSR 2650, two-component liquid silicone rubber for injection molding processes (Figure 2A).

Note: The chambers are reusable and autoclavable. Manually punch three holes in the dome of the chamber using a 2 mm biopsy punch to allow for gas exchange during the time the chamber is in place under the skin.

2. Nikon Eclipse 90i + Photometrics Cool SNAP HQ2+ DS Fi1 (Nikon, model: Ti2-E)
3. Nikon eclipse Ti-U inverted microscope (Nikon, model: Ti-U)
4. CO₂ incubator (ThermoFisher, model: HERA cell VIOS 160)
5. Straight forceps (VWR, catalog number: 82027-398)
6. Surgical scissors (VWR, catalog number: 82027-584)
7. Curved forceps (VWR, catalog number: 89259-946)
8. Pipettes P1000 (Gilson, catalog number: F144059M)
9. Pipettes P200 (Gilson, catalog number: F144058M)
10. Pipettes P20 (Gilson, catalog number: F144056M)
11. Sterile filtration system (Corning, catalog number: 431097)
12. -80 °C freezer (ThermoFisher, model: TSX Series with V-Drive)
13. Heating pad-T/Pump Professional (Gaymar, catalog number: TP700)
14. Heating pad-blanket (Cincinnati Sub-Zero, model: 274)
15. Liquid bandage New skin (Amazon, catalog number: 851409007011)

Software and datasets

1. NIS-elements Nikon (<https://www.nikoninstruments.com/Products/Software>)
2. Microsoft Excel (Microsoft, <https://products.office.com/en-us/excel>)
3. GraphPad Prism (GraphPad, <https://www.graphpad.com/scientific-software/prism/>)
4. Adobe Illustrator (<https://www.adobe.com/products/illustrator/free-trial-download.html>)

Procedure

The generation of complete grafts in this protocol spans 54 days and involves several distinct steps, as depicted in Graphical overview. In Step 1, lasting from Days 1 to 12, cryopreserved keratinocytes and fibroblasts are thawed, cultivated, and subcultured. Step 2, which occurs on Day 12, entails the surgical insertion of a silicon chamber and

the establishment of cell-seeded fibrin hydrogels. In Step 3, which takes place on Day 19, the silicon chamber is removed to promote the adherence of the graft to the surrounding tissue. Finally, in Step 4, spanning Days 19–54, the graft establishes and undergoes maturation.

The described protocol outlines the process for conducting a single surgery.

A. Defrosting and plating keratinocytes

1. Coat two 10 cm tissue culture dishes with collagen.
 - a Dilute bovine collagen stock solution (3 mg/mL) to a final working concentration of 30 µg/mL in $1 \times$ DPBS. For each 10 cm dish, resuspend 60 µL of the collagen solution in 6 mL of $1 \times$ DPBS in a 15 mL conical tube. Pipette to mix.
 - b Add 6 mL of diluted collagen solution to each 10 cm ($\sim 60 \text{ cm}^2$) tissue culture dish. Rock back and forth to ensure complete coverage.
 - c Place into the $37^\circ\text{C}/5\% \text{ CO}_2$ incubator for 1 h.
2. Prepare complete EpiLife medium (see Recipes).
3. Prewarm a 25 mL aliquot of complete EpiLife medium to room temperature (RT).
4. Aspirate non-polymerized collagen from the 10 cm dishes prepared in step A1. Immediately add 9 mL of complete EpiLife medium and transfer the dishes into a $37^\circ\text{C}/5\% \text{ CO}_2$ tissue culture incubator to equilibrate for 30 min.
5. Pipette 5 mL of EpiLife into a 15 mL conical tube. Set aside in a biosafety cabinet.
6. Remove a cryovial that contains approximately 5×10^5 frozen keratinocytes from liquid nitrogen storage and immediately place it into a 37°C water bath. Thaw quickly (approximately 1–2 min), avoiding contamination by keeping the cap above the water level.

Note: The thawing time of frozen cells depends significantly on the volume of the cells being thawed. After one minute of thawing, gently shake the vial and quickly observe the contents. If most of the cell suspension has melted, you can proceed with the next step.
7. Spray the entire vial with 70% ethanol, wipe, and proceed under strict aseptic conditions in a biosafety cabinet.
8. Transfer thawed keratinocytes into the conical tube prepared in step A5.
9. Centrifuge the cells at $200 \times g$ for 5 min at RT.
10. Gently resuspend the pellet in 1 mL of complete EpiLife medium and transfer 500 µL of the cell suspension with 2.5×10^5 keratinocytes into each 10 cm dish with the pre-equilibrated EpiLife medium prepared in step A4, to reach a proper/required plating density of $4 \times 10^3 \text{ cells/cm}^2$.
11. Gently and thoroughly disperse the cells by alternating between an up/down and then left/right motion. Repeat these motions two more times to ensure even distribution. Do not swirl the plate to mix. Once the cells are evenly dispersed, place the plate into a $37^\circ\text{C}/5\% \text{ CO}_2$ tissue culture incubator.
12. The next day, check cell attachment under a microscope.
13. Change the complete EpiLife medium every other day until cells reach approximately 60% confluency, then change daily until keratinocyte culture reaches 70%–80% confluency, at which point the cells must be passaged.

Note: If plated correctly at the density of $2.5\text{--}4 \times 10^3/\text{cm}^2$, the culture should be ready for subculturing in 5–7 days.
14. Keratinocytes must be subcultured to perform better during grafting (see below). The rest of the cells can be frozen for future experiments.

B. Subculturing keratinocytes

Note: Two 10 cm dishes with keratinocytes at 80% confluency yield approximately 2.4×10^6 cells (see Figure 1A). Since 2.0×10^6 keratinocytes are needed per surgery, two 10 cm dishes are sufficient to obtain this number of cells.

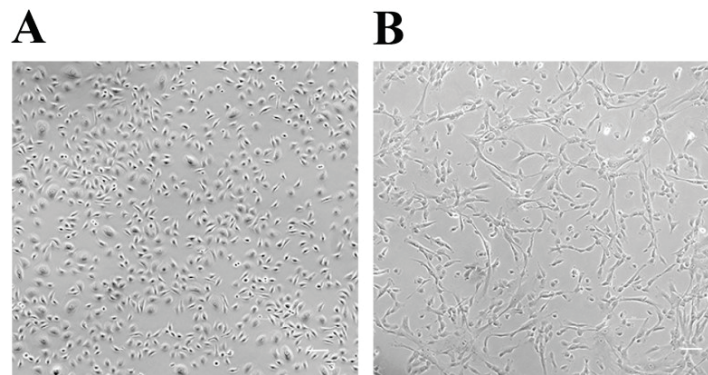


Figure 1. Representative picture of keratinocyte and fibroblast cultures ready to harvest for grafting. Keratinocytes (A) and fibroblasts (B) are shown at the appropriate confluency for grafting. Scale bar, 50 μ m.

1. Allow Accutase and complete EpiLife medium to equilibrate to RT.
2. Prepare two 10 cm tissue culture dishes and coat them with bovine collagen following the procedure described in step A1. Place into a 37 °C/5% CO₂ tissue culture incubator for 1 h.
3. After 1 h of incubation, aspirate non-polymerized collagen from the 10 cm dishes prepared above and immediately add 9 mL of complete EpiLife medium. Transfer the dishes into a 37 °C/5% CO₂ tissue culture incubator to equilibrate for 30 min.
4. Take the dish with keratinocytes that require passaging from the tissue culture incubator and aspirate spent media without disturbing the cell monolayer.
5. Add 6 mL of DPBS, gently rocking the dish to wash the cells. Aspirate DPBS.
6. Add 3 mL of Accutase and incubate at 37°C for 6–9 min.
Note: Assess the cells after 5–6 min of incubation. Many cells will start detaching or adopting a round shape morphology during this time. If more than 30% of the cells are still attached, gently tap the dish and return it to the incubator for an additional 2–3 min. After 9 min of incubation, nearly all cells should detach. It is crucial to avoid prolonged cell exposure to Accutase. Do not exceed 9 min.
Critical: If keratinocytes detach completely within only 3–4 min, this indicates that the keratinocytes have started differentiating. In this case, they should not be used for grafting; start again with earlier passage keratinocytes.
7. After incubation, tap the dish gently and add 3 mL of complete EpiLife medium to neutralize Accutase. Proceed to the next step immediately.
8. Observe under the microscope. Most cells should be floating in suspension. If not, use a wide cell scraper to collect all partially attached cells, especially from the edges of the dish.
9. Tilt the dish slightly and use a 10 mL serological pipette to gently pipette the keratinocytes one to two times, ensuring all cells are washed from the surface of the dish. Take care to avoid forming bubbles during this process.
10. Transfer the dissociated cells into a new, sterile 15 mL conical tube. Add 6 mL of DPBS to rinse away Accutase.
11. Centrifuge the cells at 200× g for 5 min at RT.
12. Aspirate supernatant. Resuspend the cell pellet in 1–5 mL of fresh EpiLife medium and count cells using a hemocytometer.
13. Transfer $2.5\text{--}4.0 \times 10^3$ keratinocytes per cm² into each collagen-coated 10 cm dish with pre-equilibrated EpiLife medium prepared in step B3.
14. Gently and thoroughly disperse the cells by alternating between an up/down and then left/right motion. Repeat these motions two more times to ensure even distribution. Do not swirl the plate to mix. Once the cells are evenly dispersed, place the plates into a 37 °C/5% CO₂ tissue culture incubator and leave the plates undisturbed for at least 24 h to allow the cells to attach to the surface of the dish.
15. The next day, examine cell attachment under a microscope.

16. Change complete EpiLife medium every other day until the keratinocyte culture reaches approximately 60% confluency. Once it reaches this density, change the medium daily until the culture reaches 70%–80% confluency. At this point, the cells are ready to be passaged.

Note: If keratinocytes are plated at a density of $2.5\text{--}4 \times 10^3$ cells/cm², the culture should be ready for subculturing in 4–5 days (see Figure 1A).

C. Defrosting and plating fibroblasts

1. Prepare complete FEM medium as described above (see Recipes).
2. Prewarm a 25 mL aliquot of complete FEM medium to RT.
3. Add 9 mL of complete FEM medium into two 10 cm dishes and transfer them into a 37 °C/5% CO₂ tissue culture incubator to pre-equilibrate for 30 min.
4. Pipette 5 mL of complete FEM medium into a 15 mL conical tube. Set aside in a biosafety cabinet.
5. Remove the cryovial containing approximately 5×10^5 frozen fibroblasts from liquid nitrogen storage and immediately place it into a 37 °C water bath. Thaw quickly (approximately 1–2 min), taking care to avoid contamination by keeping the cap above the water level.

Note: Thawing time depends on the volume of frozen cells. After one minute, shake the vial and quickly observe the contents. If most of the cell suspension has melted, you can proceed with the next step.

6. Spray the entire vial with 70% ethanol, wipe, and proceed under strict aseptic conditions in a biosafety cabinet.
7. Transfer thawed fibroblasts into a conical tube with 5 mL of complete FEM medium prepared in step C4.
8. Centrifuge the cells at $200\times g$ for 5 min at RT.
9. Gently resuspend the pellet in 1 mL of complete FEM medium and transfer 500 µL of the cell suspension with 2.5×10^5 fibroblasts into each 10 cm dish with the pre-equilibrated FEM medium prepared in step C3, to reach a proper/required plating density of 4×10^3 cells/cm².
10. Gently and thoroughly disperse the cells by alternating between an up/down and then left/right motion. Repeat these motions two more times to ensure an even distribution. Do not swirl the plate to mix. Once the cells are evenly dispersed, place the plate into a tissue culture incubator and leave it undisturbed for at least 24 h to allow the cells to attach to the dish.
11. The next day, examine cell attachment under a microscope.
12. Change complete FEM medium every other day until the cells reach approximately 80% confluency. Once they reach this density, passage the cells.

Note: If fibroblasts are cultured in FBS-containing medium, they should be transitioned into FEM medium (see Recipes, Table 1). Culturing fibroblasts in complete FEM medium for four days is sufficient for a successful transition, which will result in changes in fibroblast phenotype and accelerated growth of these cells, as shown in Figure 1B.

13. Fibroblasts must be subcultured to perform better during grafting (see below). The rest of the cells can be frozen for future experiments.

D. Subculturing fibroblasts

Note: One 10 cm dish with fibroblasts at 85% confluency yields approximately 2.5×10^6 cells (see Figure 1B). Since 2.0×10^6 fibroblasts are needed per surgery, one 10 cm dish is sufficient to obtain this number of cells.

1. Prepare one 10 cm tissue culture dish and allow Trypsin-EDTA and complete FEM medium to equilibrate to RT.
2. Add 9 mL of complete FEM medium to the dish and transfer it into a 37 °C/5% CO₂ tissue culture incubator to equilibrate for 30 min.
3. Take the dish with fibroblasts that require passaging from the tissue culture incubator and aspirate spent media without disturbing the cell monolayer.
4. Add 10 mL of DPBS, gently rocking the dish to wash the cells. Aspirate DPBS.
5. Add 3 mL of Trypsin-EDTA and incubate at 37 °C in a tissue culture incubator for 2–3 min.
6. After incubation, tap the dish gently and add 6 mL of complete FEM medium to neutralize Trypsin-EDTA.

7. Observe the cells under a microscope. Most cells should be floating in suspension. Alternatively, use a wide cell scraper to collect all partially attached cells, especially from the edges of the dish.
8. Tilt the dish and gently pipette with a 10 mL serological pipette one to two times to wash all fibroblasts from the surface of the dish. Avoid forming bubbles.
9. Transfer the dissociated cells into a new, sterile 15 mL conical tube. Add 3 mL of DPBS to dilute Trypsin-EDTA.
10. Centrifuge cells at $200\times g$ for 5 min at RT.
11. Aspirate supernatant. Resuspend the cell pellet in 1–5 mL of fresh complete FEM medium and count cells using a hemocytometer.
12. Transfer 2.5×10^3 fibroblasts per cm^2 to the dish with pre-equilibrated FEM medium prepared in step D2.
13. Gently and thoroughly disperse the cells by alternating between an up/down and then left/right motion. Repeat these motions two more times to ensure even distribution. Do not swirl the plate. Once the cells are evenly dispersed, transfer the plate into a $37^\circ\text{C}/5\% \text{CO}_2$ tissue culture incubator and leave the plate undisturbed for at least 24 h to allow the cells to attach to the dish.
14. The next day, check cell attachment under a microscope.
15. Change the complete FEM medium every other day until cells reach approximately 70%–80% confluency (Figure 1B).
Note: If fibroblasts are plated at the density of $2.5 \times 10^3/\text{cm}^2$, the culture should be ready for subculturing in 3 days.

E. Preparation of working solutions and cells for grafting (for one graft)

1. Defrost thrombin stock solution (25 U/mL) overnight on ice at 4°C .
2. Defrost the fibrinogen aliquot in a fridge for approximately 1 h before use.
3. On the day of the surgery, prepare the working Solution 1 and Solution 2 according to Tables 3 and 4 (see Recipes for working solutions). Immediately place all components on ice while preparing cells and mice for surgery.
4. Collect expanded keratinocytes and fibroblasts from section B and section D, respectively.
Note: It is important that both keratinocytes and fibroblasts are of good quality and within 75% and 85% confluency, respectively, on the day of surgery (Figure 1A–1B). The use of differentiated or senescent cells will result in graft failure. Collect cells immediately before the surgery.
 - a. Collect 2.0×10^6 fibroblasts as described in steps D1–D10, count cells using a hemocytometer, and centrifuge the cells to obtain a pellet. Resuspend the pellet in 50 μL of complete FEM medium and put the tube with resuspended fibroblasts on ice. Promptly use for surgery to preserve cell viability.
 - b. Collect 2.0×10^6 keratinocytes as described in steps B1–B11, count cells using a hemocytometer, and centrifuge the cells to obtain a pellet. Resuspend the pellet in 50 μL of complete EpiLife medium and put the tube with resuspended keratinocytes on ice. Promptly use for surgery to preserve cell viability.
5. (Optional) If frozen-then-thawed keratinocytes and fibroblasts are grafted, follow the procedure below (described for one graft):
 - a. Thaw 2.0×10^6 keratinocytes and 2.0×10^6 fibroblasts as described in steps A6–A9 and C5–C8, respectively. Count the cells before centrifuging to ensure that the correct number of viable cells are used.
 - b. Carefully resuspend the pellet containing thawed fibroblasts in 50 μL of complete FEM medium and the pellet containing thawed keratinocytes in 50 μL of complete EpiLife medium. Keep the resuspended cells on ice and promptly use them for surgery to preserve cell viability.
Note: It is crucial to freeze and thaw cells under optimal conditions to ensure cell viability and functionality. See the supplementary information below.
6. Place the following on ice to transport to the animal facility for grafting. All volumes shown below are for one graft:

• Solution 1	323.3 μL
• Suspension of fibroblasts	50 μL
• Solution 2	323.3 μL

- Suspension of keratinocytes 50 μ L
- Fibrinogen (40 mg/mL stock) 50 μ L
- Thrombin (25 U/mL stock) 25 μ L

F. Grafting procedure

Follow the protocol for the insertion of a silicone chamber into a nude mouse as outlined in Diette et al. (2020) or refer to Figure 2B–2D and Video 1. Briefly:

1. Administer anesthesia to the mice via an intraperitoneal injection with prepared anesthetic solution in adherence to animal protocol.
2. Wipe the back skin of the anesthetized mouse with an alcohol swab and let the skin dry.
3. Use forceps to pinch and elevate the skin above the flat area of the back. Cut a piece of the skin with curved scissors to create a round incision, approximately 1 cm in size (Figure 2B).
4. Apply a 200 μ L drop of sterile DPBS with antibiotics onto the opened muscle fascia to provide lubrication. Gently release a rim of the skin surrounding the incision (approximately 5 mm) by lifting the edges with forceps. Simultaneously, glide along the rim of the incision with a 1 mL tip, separating the skin from the underlying muscle fascia.
5. Using straight forceps, pull up the edge of the skin near the incision, creating an elongated slit-like opening between the skin and muscle fascia. Simultaneously, grasp a silicone chamber with curvy forceps and slightly squeeze the dome of the chamber.
6. Slide one side of the chamber's rim beneath the skin on one side of the slit (Figure 2C).
7. With forceps, gently pull the skin over the chamber's rim on the other side of the slit simultaneously releasing the curvy forceps. Release both forceps and secure the entire lower rim of the chamber beneath the skin by ensuring that the rim is flat under the skin (Figure 2D).
8. After the chamber has been successfully inserted, transfer the mouse to a clean cage placed on a heating pad **without a wire feeder**.
9. Proceed promptly with the cell transplantation procedure as described in section G.

Critical: Ensure that the wound bed inside the implanted chamber does not dry during the procedure.

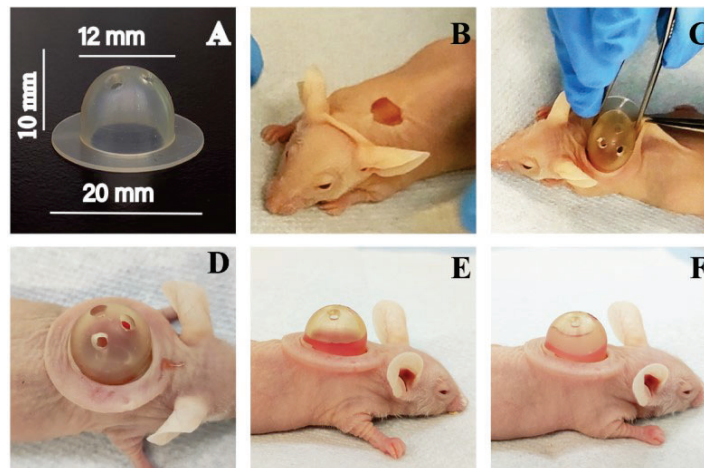
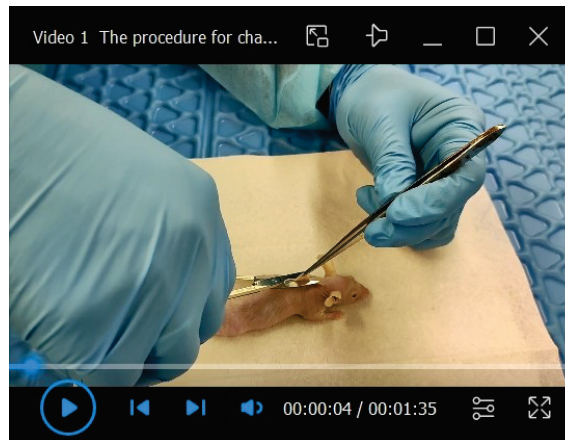


Figure 2. Visual representation of the grafting procedure. (A) Dimensions of the silicone chamber are shown. (B) The mouse skin is cut to create a hole with a circumference of approximately 1 cm for chamber implantation. The chamber is then inserted into the wound (C–D), and a fibroblast-comprised fibrin hydrogel is delivered to form the dermal layer (E). Following the polymerization of the dermal layer, a keratinocyte-comprised fibrin hydrogel is delivered on top (F).



Video 1. Procedure for chamber insertion

G. Delivery of the gel containing fibroblasts

Keep all tubes with working solutions, fibrinogen, thrombin, and cell suspensions on ice before use. Spray the vials with 70% ethanol, wipe them, and proceed under strict aseptic conditions in a biosafety cabinet or a clean procedure room in an animal facility.

Critical: To maintain the integrity and viability of the samples, it is imperative to perform all steps expeditiously, ensuring that reagents and materials are kept chilled on ice throughout the procedure.

1. Using a pipette, transfer the entire volume of cold working Solution 1 (323.3 μ L) into the tube containing the suspended fibroblasts prepared in step E4a (or E5 if thawed cells are used). Gently mix the contents, taking care to avoid the formation of bubbles.
2. Immediately before transferring into an implanted chamber, add 9.4 μ L of 25 U/mL thrombin and 17.3 μ L of 40 mg/mL fibrinogen to the cell suspension from step G1. Resuspend gently by pipetting 2–3 times, avoiding bubbles (see Table 3).
3. Immediately transfer the final mix (400 μ L) into the chamber using a narrow-tip 1 mL pipette.
4. Gently shake the mouse to facilitate the distribution of the hydrogel solution throughout the wound bed.
Note: With secure and proper chamber placement, the fibrin gel should be slightly visible in the lower portion of the silicone chamber (Figure 2E).
5. Place the mouse in the cage on a warming pad and allow it to remain there for a period of 15 min. This timeframe allows the fibrin hydrogel to undergo polymerization, resulting in the formation of a dermal layer. It is important to provide sufficient time for the hydrogel to solidify and establish the desired structural integrity.

H. Delivery of the gel containing keratinocytes

While the dermal layer solidifies, prepare the epidermal fibrin gel containing keratinocytes.

1. Using a pipette, transfer the entire volume of cold working Solution 2 (323.3 μ L) into the tube containing the keratinocyte suspension prepared in steps E4b (or E5 if thawed cells are used). Gently mix the content, taking care to avoid the formation of bubbles.
2. Immediately before transplanting into an implanted chamber with the dermal layer, add 9.4 μ L of 25 U/mL thrombin and 17.3 μ L of 40 mg/mL fibrinogen to the cell suspension. Resuspend gently by pipetting 2–3 times, avoiding bubbles (Table 4).
3. Immediately transfer the final mixture (400 μ L) into the chamber on top of the solidified fibrin gel containing fibroblasts.

Note: Avoid pulling or tilting the silicon chamber when transferring the mixture. It is important to handle the chamber carefully to prevent any disruption or displacement of the grafted material.

4. Gently shake the mouse to ensure even distribution of the hydrogel-containing keratinocytes over the solidified hydrogel containing fibroblasts previously transferred into the silicone chamber.

Note: With secure and proper chamber placement, the hydrogels should be visible and fill one-half to three-fourths of the silicone chamber (Figure 2F).

5. Place the mouse in the cage on a warming pad and allow it to remain there for a period of 15–40 min to let the fibrin gel polymerize to create the epidermal layer.

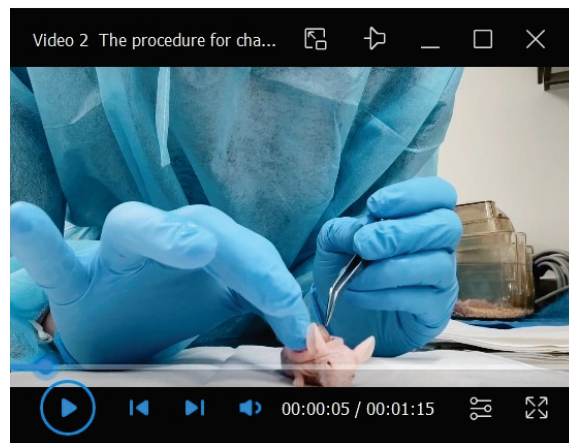
Critical: Ensure that the mouse remains calm for approximately 15 min to allow each layer to polymerize. Additionally, after applying both fibrin layers, keep the mouse undisturbed for at least 1 h. Consequently, the most optimal duration for anesthesia is 2 h.

6. Keep the chamber on mice for seven days.

Note: During the first 10 days post-surgery, check the mice regularly and provide them with moist chow daily. This helps to ensure the mice are hydrated and supports their post-operative recovery.

I. Chamber removal, wound healing, and skin graft maturation

On day 7 post-surgery (Day 19), remove the chamber from grafted mice as described in Diette et al. (2020) or refer to Video 2. Briefly:



Video 2. Procedure for chamber removal

1. Administer anesthesia to the mice via an intraperitoneal injection with prepared anesthetic solution in adherence to animal protocol.
2. Wipe the skin around the chamber and the dome of the chamber with an alcohol swab.
3. Carefully loosen the skin around the chamber with sterile forceps.
4. With a clean, gloved finger, gently squeeze the dome of the silicone chamber and carefully pull one side from under the skin. Carefully release one edge of the rim using forceps.
5. Ensure that the graft is not attached to the inner part of the chamber and the forming skin graft stays in the wound.

Notes:

- a. *If the edge of the graft and connective tissues around it remain attached to the silicone chamber, run another pair of forceps along the silicone lip of the chamber to free the graft.*
- b. *The resulting graft should appear as an off-white plug with no yellow areas, which indicates minimal tissue decay (Figure 3A–3D). The wound surface can exhibit varying levels of dryness, which does not significantly affect graft formation.*

6. Apply New skin liquid bandage onto the mouse skin around the open wound to prevent mouse skin contraction.
7. Place the mouse into a clean cage (without a wire feeder) that has been resting on a heating pad for at least 1 h. After the mouse recovers from anesthesia, remove the heating pad and monitor the mouse for the duration of the healing (approximately six weeks after surgery).

Note: The scab that forms after chamber removal will naturally detach within 2–2.5 weeks.

8. Harvest the graft for histological analysis or live in vivo studies six weeks after surgery (day 54 of the protocol).

Note: The size of the human skin grafts after chamber removal is constrained by the chamber dimensions and ranges from 1.0 to 1.5 cm in diameter (Figure 3A–3D). Following healing, the grafts typically measure between 0.4 and 1.2 cm in diameter (Figure 3E–3H). This size is suitable for various applications, such as assessing skin cell functionality, modeling the phenotype of skin disorders, investigating wound healing stages, studying aging, and evaluating skin barrier function. The graft can be analyzed earlier, at five weeks post-surgery, if necessary.

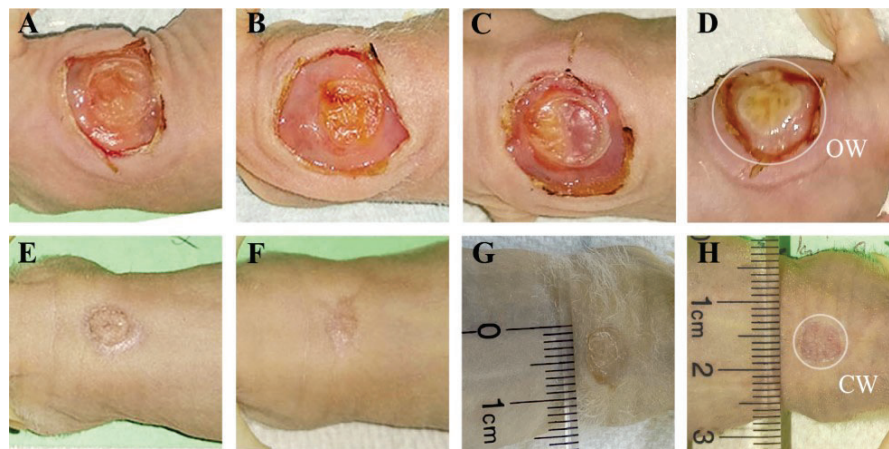


Figure 3. Representative images of grafts at different stages of the healing process. The fibrin-based grafting was performed with (A, E) 2.0×10^6 freshly cultured keratinocytes together with 2.0×10^6 of freshly cultured fibroblasts; (B, F) 1.0×10^6 freshly cultured keratinocytes together with 1.0×10^6 freshly cultured fibroblasts, and (C, G) 2×10^6 frozen-then-thawed keratinocytes together with 2×10^6 frozen-then-thawed fibroblasts. As a control, grafts generated using a classical grafting chamber assay with 5.0×10^6 freshly cultured keratinocytes and 5.0×10^6 freshly cultured fibroblasts are shown (D, H). Representative images of forming grafts on the day of chamber removal (day 7) are shown on top and corresponding fully established grafts at 5 weeks post-grafting are shown on the bottom. OW—opened wound after chamber removal, and CW—closed wound at the day of harvest of the skin graft.

J. Histological examination of human skin xenografts

1. Euthanize the mouse on the day of harvesting (Day 54) in accordance with the approved protocol by the Institutional Animal Care and Use Committee (IACUC).
2. Use a permanent marker to mark the borders of the graft and carefully cut the mouse skin around the graft, maintaining a 5 mm distance from the edge of the graft.
3. Gently peel off the skin and transfer it to Whatman paper to ensure that it lays flat.
4. Fix sections with skin grafts in a buffered solution of 10% Formalin for 24 h and then transfer them into 70% ethanol.
5. Fixed tissue can be paraffin-embedded and sectioned for further histological analysis. If histological analysis is performed, prepare 7 μ m sections that include both the center and edge portions of the graft.
6. Confirm the presence of the human skin graft through a conventional histological analysis of tissue

sections stained with hematoxylin and eosin (H&E) (Yanez et al., 2015). In this staining method, the epidermis shows up as a thin, compact multilayer structure on the top of the skin, exhibiting a dark pink color with a stratum corneum overlay (Figure 4A). The dermis appears as a light-pink, substantial layer beneath the epidermis, featuring nuclei stained in purple. Notably, a human graft typically displays a thicker stratum corneum and epidermis compared to adjacent mouse skin (Figure 4A, 4D, 4G, and 4J).

7. Conduct immunohistochemical analysis following the protocol outlined in Yanez et al. (2015). To label the epidermis, use the anti-K14 antibody (BioLegend 906004) at a 1:2000 dilution. Co-staining with the anti-mouse K1 antibody (BioLegend 905602) at a 1:500 dilution aids in delineating human graft borders (refer to Figure 4B, 4E, 4H, and 4K, where K14 is represented in red and K1 is in green). For visualizing the human dermis, employ a human specific anti-Vimentin antibody (ab16700) at a 1:500 dilution. When co-stained with the anti-human Loricrin antibody (ab176322) at a 1:2000 dilution, the human Vimentin staining provides insights into the proper localization of the human dermis beneath the human epidermis (see Figure 4C, 4F, 4I, and 4L, with Vimentin represented in red and Loricrin in green). Use corresponding secondary antibodies at a 1:300 dilution, as listed in the reagents section. Capture images using a Nikon Eclipse 90i microscope equipped with a Photometrics Cool SNAP HQ2+ DS Fi1 Nikon confocal system or another comparable microscope system.

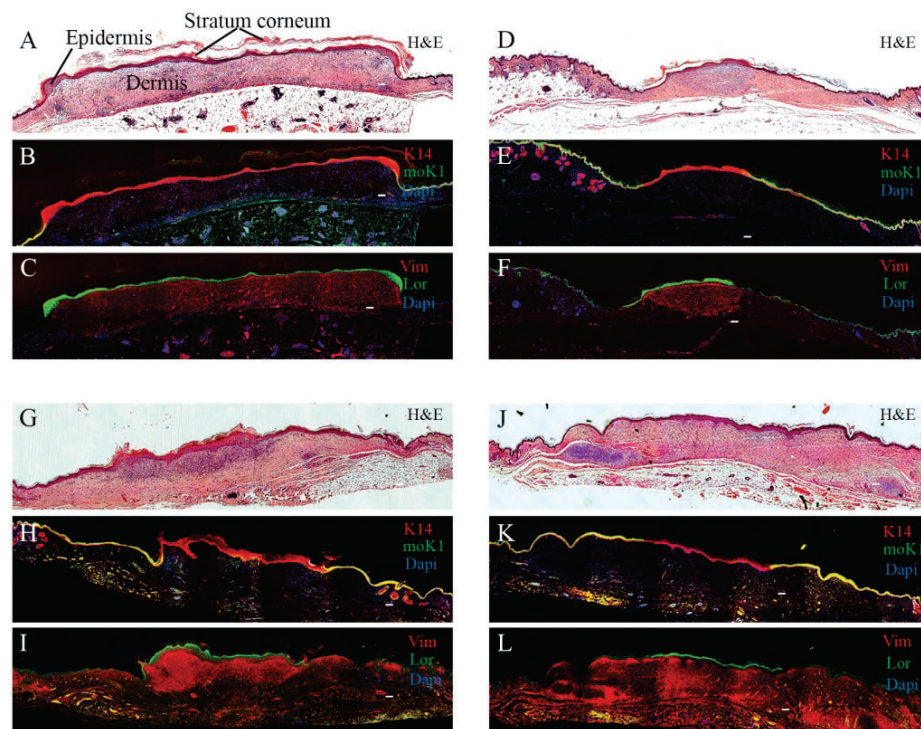


Figure 4. Histological examination of human skin xenografts. Representative images of fibrin-based grafts generated with 2.0×10^6 freshly cultured keratinocytes together with 2.0×10^6 freshly cultured fibroblasts (A–C), 1.0×10^6 freshly cultured keratinocytes together with 1.0×10^6 freshly cultured fibroblasts (D–F), as well as with 2.0×10^6 frozen-then-thawed keratinocytes together with 2.0×10^6 frozen-then-thawed fibroblasts (G–I) are shown. As a control, grafts generated using a classical grafting chamber assay with 5.0×10^6 freshly cultured keratinocytes and 5.0×10^6 freshly cultured fibroblasts were also analyzed (J–L). H & E staining of the grafts (A, D, G, J). Immunofluorescence staining using mouse-specific Keratin (K)1 (moK1; green) and human/mouse-specific K14 (red). Note that the moK1 antibody stains only the mouse epidermis (B, E, H, K). Immunofluorescence staining using human-specific Loricrin (Lor; green) detects the human epidermis, while human-specific Vimentin (Vim, red) detects the human dermis (C, F, I, L). Nuclei stained with DAPI (blue). Scale bar, 100 μ m.

Data analysis

To assess the quality of the grafts, several parameters can be assessed such as (1) the length and thickness of the developed human skin graft and (2) the thickness of the epidermis and dermis using histological analysis and immunofluorescence staining. To compare the means of graft measurements between the grafted groups, conduct an ANOVA test (GraphPad Prism).

Graft measurements

A. Wound contraction

1. Using a digital camera and a sterile ruler calibrated in centimeters placed laterally underneath the graft, capture an image of the opened wound (OW) on Day 19 after chamber removal and the closed wound (CW) on Day 54 before graft harvesting (Figure 3D and 3H).
2. Utilizing the *Measure* tool in Adobe Illustrator, ascertain the number of pixels equivalent to 1 centimeter on the ruler within the photograph. Establish a pixel-to-centimeter conversion.
3. Determine the number of pixels representing the diameter of the OW and the CW.
4. Recompute the wound diameters into centimeters, using the pixel-to-centimeter conversion established earlier.
5. Divide the calculated wound diameter in two to find the radius of the wound (r).
6. Recalculate the wound area (cm^2) following:

$$\text{Wound area} = \pi r^2$$

7. Define the percentage of wound contractions as follows:

$$\% \text{ of wound contraction} = \left(1 - \frac{\text{Closed wound area}}{\text{Opened wound area}}\right) \times 100$$

B. Skin layers measurement

1. Use NIS-elements Nikon Software to merge colors and set the 100 μm scale bar (Figure 4).
2. Utilize the *Measure* tool in Adobe Illustrator to measure the size of the human graft and the thickness of the epidermis and dermis based on immunohistochemical staining of skin layers.

Validation of protocol

To establish this grafting method, a total of 28 grafting procedures were performed. Eight grafting procedures were performed with 2×10^6 freshly cultured keratinocytes together with 2×10^6 freshly cultured fibroblasts with a 100% success rate. When the number of cultured cells decreased, the success rate of grafting decreased to 80%. Specifically, five grafting procedures were performed with 1×10^6 freshly cultured keratinocytes together with 1×10^6 freshly cultured fibroblasts, and four grafts successfully formed. Importantly, a typical success rate of a classical chamber grafting assay that utilizes 5×10^6 of freshly cultured keratinocytes together with 5×10^6 of freshly cultured fibroblasts also fluctuates between 60% and 80% (Wang et al., 2000; Diette et al., 2020). Therefore, our grafting approach allows for more consistent human skin graft formation with fewer cell numbers compared to other methods of skin grafting.

Interestingly, when 2×10^6 frozen-then-thawed keratinocytes together with 2×10^6 frozen-then-thawed fibroblasts were used for grafting, our approach resulted in a 72% success rate (8 successful engraftments out of 11 grafts), providing feasibility data for using frozen-then-thawed skin cells in skin transplantation. Additionally, as a control, four classical skin grafting chamber assays were also performed.

The procedure described in this manuscript is currently being used in our laboratory in at least three different projects and is reproducible among multiple laboratory members.

The quality of the human graft, particularly the size and thickness of the epidermis and dermis, are important factors that influence graft persistence and usefulness in downstream applications. We plotted different measurements of the grafts generated under different conditions and found significant differences in the parameters of the resulting grafts (Figure 5A–5D). Four grafts from each group were analyzed in week 6 after surgery.

First, we analyzed the contraction of the wound area during graft healing. The extent of mouse skin contraction can significantly impact the size of resulting human xenografts once they have healed. Our experimental data revealed that grafts created with 2.0×10^6 freshly cultured keratinocytes together with 2.0×10^6 freshly cultured fibroblasts exhibited wound contraction of $52.8\% \pm 11.2\%$, while grafts generated with 1.0×10^6 cultured keratinocytes together with 1.0×10^6 freshly cultured fibroblasts demonstrated a contraction of $88.7\% \pm 3.7\%$. The utilization of 2×10^6 frozen-then-thawed keratinocytes together with 2×10^6 frozen-then-thawed fibroblasts resulted in the contraction of $62\% \pm 5.2\%$. In comparison, the control graft produced using the classical chamber assay performed with 5.0×10^6 cultured keratinocytes together with 5.0×10^6 cultured fibroblasts exhibited a contraction rate of $60.9\% \pm 4.7\%$ (Figure 5A). The accuracy of graft size measurements was affirmed through concurrent histological analysis, which showed that the grafts generated with 2.0×10^6 freshly cultured keratinocytes together with 2.0×10^6 freshly cultured fibroblasts exhibited the largest surface area (Figure 4A–4C and Figure 5B).

The reported contraction rates of our grafts were in alignment with other reports that utilized other hydrogel-based methods (Del Rio et al., 2002; Llamas et al., 2004; Kalyanaraman and Boyce, 2009; Martinez-Santamaria et al., 2012; Yanez et al., 2015; Jorgensen et al., 2020). For instance, Kalyanaraman and Boyce (2009) reported a contraction rate of approximately 64%–70% in the grafts generated with a 4 cm² fibroblast-populated collagen-glycosaminoglycan sponge as the dermal component (Kalyanaraman and Boyce, 2009). Another study demonstrated a 50% contraction rate by week 3 when a 6.25 cm² wound was covered with a three-layered fibrin-based bio-printed skin (Jorgensen et al., 2020). Similarly, 3D-printed collagen-based grafts exhibited a 62.5% contraction rate by week 6 when applied to a 2.5 cm² wound (Yanez et al., 2015).

The thickness of the graft is crucial as it affects the degree of shrinkage within the wound bed over time and influences the diffusion of nutrients from the wound bed during the initial healing stages. We measured the thickness of the dermis and epidermis in our grafts based on histological analysis. The average dermal layer thickness within our grafts ranged from 314.2 to 621.4 μm, which was on the lower end of dermal thickness in the native human skin, with no significant differences between grafting variants (Figure 5C). The thickness of the epidermis ranged between 34.2 and 92.3 μm, which was also on the lower end of the epidermal thickness of native human skin (Lintzeri et al., 2022). The only deviation from this number was observed in grafts generated with 2×10^6 frozen-then-thawed keratinocytes together with 2×10^6 frozen-then-thawed fibroblasts. These grafts developed a thicker epidermis, which ranged from 128 to 140 μm (Figure 5D). The differences in the parameters of the grafts generated with frozen-then-thawed cells vs. those produced with freshly cultured cells probably result from functional alterations during the freezing and thawing processes.

The observed differences in graft characteristics highlight the importance of cell numbers and processing methods in achieving desired outcomes in skin grafting procedures.

In summary, grafts generated with 2.0×10^6 freshly cultured keratinocytes and 2.0×10^6 freshly cultured fibroblasts were reproducible and of superior quality. Therefore, this composition emerges as the most optimal variant within the grafting protocol delineated in this study. Grafts made with frozen-then-thawed keratinocytes and fibroblasts can also serve as an appropriate alternative if needed.

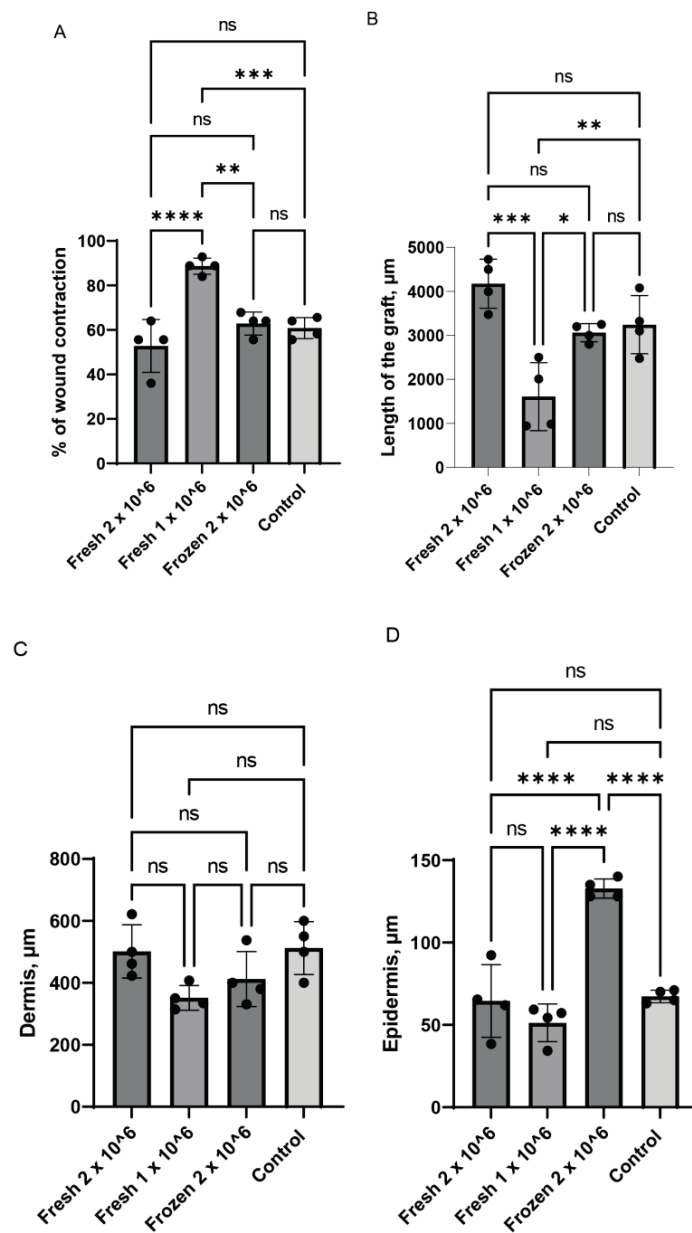


Figure 5. Digital planimetry. Individual plots for a wound contraction rate are shown in A. The length of grafts (B), the thickness of the dermis (C), and the thickness of the epidermis (D) were determined by analyzing immunofluorescence images of grafts developed at 6 weeks after surgery (**** $p < 0.0001$; *** $p < 0.002$; ** $p < 0.0094$; * $p < 0.02$).

General notes and troubleshooting

General notes

1. For the first time, our study reports the successful layer-by-layer engraftment of skin cells *in vivo*. It shows the feasibility of transplanting layered skin without the need to perform 3D skin bioprinting or generate complex 3D skin equivalents in a dish, both labor intensive and requiring specialized equipment (Cooper et al., 1993; Jorgensen et al., 2020). Therefore, with further modifications, the provided method may serve as a foundation for new clinical protocols for skin transplantation. Our protocol is expected to yield superior outcomes compared to grafting approaches involving keratinocyte sheets, semi-split, or fragile full-thickness grafts.
2. Our protocol is specifically optimized for the use of human neonatal keratinocytes and fibroblasts on passage two. However, it can be tailored to skin cells collected at later passages (up to passage four for keratinocytes and up to passage six for fibroblasts) and of different origins (data not shown).
3. Our protocol also allows for the successful engraftment of frozen-then-thawed keratinocytes and fibroblasts (Figures 3C and 3G). This is the first report that shows the successful generation of *in vivo* full-thickness skin grafts using frozen-then-thawed skin cells without prior cell culture expansion. Since the transportation and storage of frozen cells are more accessible than *in vitro*-produced skin equivalents, the feasibility of transplanting frozen skin cells may dramatically simplify skin transplantation in burn patients and patients with chronic wounds.
4. The human skin grafts generated with this protocol persist for at least three months after grafting onto immunocompromised mice.
5. Human fibrin-based hydrogels are commonly used as a matrix for tissue bioengineering and skin transplantation and are FDA-approved. To ensure the clinical applicability and success of our method, we use highly pure and cell culture-tested human plasma-derived fibrinogen and thrombin in this protocol.
6. In our modified chamber assay, the final concentration of fibrinogen is 1.73 mg/mL and the thrombin concentration is 0.59 U/mL. These concentrations are significantly lower than the recommended ranges for clinical and research applications, where fibrinogen concentrations typically range from 5 to 80 mg/mL and thrombin concentrations range from 1 to 125 U/mL. However, the concentrations used in our formulation produce soft gels that solidify within 15 min. The soft gel structure has relatively large pore sizes that facilitate cell migration within the gel. In addition, the presence of abundant surrounding medium promotes cell viability. The final stiffness of the gel does not affect the graft size, and the gel effectively covers the entire wound area. Therefore, these conditions are likely to enhance cell viability, support cell division and migration, and facilitate cellular rearrangement in a favorable environment.
7. A potential limitation of our proposed method is the time required to form hydrogel-encapsulated cells. Compared to the standard chamber assay, creating dermal and epidermal layers in our approach results in a 15-min delay in transferring cells from tissue culture dishes to the wound surface. However, this delay does not affect cell viability, and our method results in less contraction of the grafts (52.8%) compared to the standard chamber assay (60.1%).

Troubleshooting

Graft failure. The quality of keratinocytes is critical for successful engraftment. Preventing keratinocytes from reaching a confluency level higher than 75% is essential. Exceeding this threshold can lead to terminal differentiation of the cells, impeding their effective engraftment. Similarly, fibroblasts should not be allowed to reach 85% confluency before using them for surgery, as they should be mitotically active at the time of grafting. Make at least three biological repeats.

The number of fibroblasts is insufficient for surgery. The dermal component can be successfully generated with fewer than 1.0×10^6 fibroblasts without compromising the reproducibility of the method (data not shown).

The number of keratinocytes is insufficient for surgery. Our protocol can be performed with as low as 0.5×10^6 cultured keratinocytes and 0.5×10^6 cultured fibroblasts. However, the success rate of these grafts is lower, approximately 40% (data not shown).

Frozen cells do not engraft as efficiently as freshly cultured cells. It is crucial to freeze and thaw cells properly for their successful engraftment. Our protocol uses Cryo50 (ZenBio) for cryopreserving keratinocytes and CryoStor CS10 (StemCell Technologies) for fibroblasts. The freezing and thawing procedures are conducted according to the manufacturer's recommendations, which are further described in Supplementary Materials.

The fibrin gel adheres to the silicon chamber during the grafting procedure. During the grafting procedure, one potential challenge is the adhesion of the fibrin gel to the silicon chamber's walls. To prevent this, ensure that the chamber is clean and free of debris or moisture before introducing the fibrin gel. Avoid overloading the chamber and follow the protocol's recommended volumes of cell-loaded gels. Overfilling the chamber can lead to excess gel contacting the walls, increasing the likelihood of gel adhesion. Following the protocol diligently and using the specified volumes of gel solutions will contribute to the overall success of the procedure and minimize any issues related to the adherence of the gel to the chamber walls.

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Competing interests

DRR has an equity interest in AVITA Medical. KB is an employee of AVITA Medical, and AH was an employee at the time the research was conducted. AVITA Medical may potentially benefit from the research findings presented here. Other authors do not have any potential conflicts of interest to declare.

Ethical considerations

All animals were housed and handled in accordance with the Institutional Animal Care and Use Committee (IACUC) of The University of Colorado Anschutz Medical Campus. All studies were performed with the approval of the IACUC committee.

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Supplementary information

The following supporting information can be downloaded [here](#):

1. Supplementary information

APPENDIX 4

Manuscript: Warshauer et al., 2024

Title

Secret Sephardic Origins Revealed for Rare Skin Disorder, Recessive Dystrophic Epidermolysis Bullosa, in Individuals Carrying the Unique c.6527insC Mutation

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Epidermolysis Bullosa, Genodermatoses, Jewish Population Genetics

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Conflict of Interest

The authors state no conflict of interest.

Abstract

Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare and severe blistering skin disorder caused by mutations in the type VII collagen gene (*COL7A1*). The *COL7A1* c.6527insC mutation is curiously prevalent amongst RDEB individuals. Previous research suggested the possibility of a Sephardic Jewish origin of the mutation, however RDEB individuals are not known to have predominant Jewish ancestry. In this study, a global cohort of 126 RDEB individuals with the c.6527insC founder mutation from Spain, France, Argentina, Chile, Colombia, and the USA were investigated. Sephardic ancestry was identified at the haplotype spanning the c.6527insC mutation in 85% of the individuals, despite mixed ancestry elsewhere in the genome and no known recent Sephardic ancestry. An age estimation analysis of the c.6527insC mutation was performed and identity-by-descent matching between this RDEB subpopulation and a known crypto-Jewish community in Belmonte, Portugal was ascertained, providing support for crypto-Jewish ancestry in this unique RDEB subpopulation.

Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare and severe blistering skin disorder caused by mutations in the type VII collagen gene (*COL7A1*).¹⁻³ RDEB exhibits pronounced mutational heterogeneity and most *COL7A1* mutations recur quite infrequently,⁴ yet the *COL7A1* c.6527insC mutation is intriguingly prevalent.⁵ The recent investigation of several RDEB Hispanic populations from Spain and other Spanish-speaking populations in Europe and the Americas revealed common ancestry, inherited at least in part through Sephardic migration, among individuals carrying the c.6527insC mutation.⁶

Jewish people have faced complex waves of migrations during their extensive and dynamic history, resulting in elaborate population genetic patterns, and providing insight into general patterns of health and disease in society.^{7,8} The majority of Sephardic Jews emigrated from Spain during the time of the Spanish Inquisition to other European countries, North Africa, the Middle East and the Americas, along with a number of pathogenic mutations. The c.6527insC mutation in modern Jews originally dates to ~1300 BCE with successive admixture amongst ancient Iberians occurring in ~900 CE and with Native Americans ~1492 CE (Fig. 1a and 1b). It is still present in Spain today. Many who did not leave Spain during the Inquisition converted to Catholicism while continuing to observe Judaism in secrecy, creating crypto-Jewish communities. The presence of surviving crypto-Jewish descendants has been established in an earlier study of paternal lineages across the Iberian Peninsula.⁹

RDEB individuals carrying the c.6527insC mutation with otherwise unknown Jewish ancestry from Spain, France, Argentina, Chile, Colombia, and the USA were investigated in detail and considerable Sephardic ancestry at the region of the c.6527insC mutation was unambiguously identified. The findings in this study strongly support crypto-Jewish roots as part of RDEB history.

Results

Local ancestry analysis

myOrigins v3.0 analysis found a strong signal of Sephardic population ancestry spanning the c.6527insC mutation (Fig. 2). Out of 164 haplotypes carrying the mutation (38 homozygotes; 88 heterozygotes), 136 (83%) were found to be Sephardic, and 142 (87%) were found to be Jewish more generally. In contrast, only 15% (95% CI: 8–22%) of the entire genome was Sephardic, and only 22% (95% CI: 13–30%) of the entire genome was Jewish (yellow line in Fig. 2b). Many heterozygotes did not have overlapping Sephardic ancestry on their second haplotype (Fig. S1), which included a range of other RDEB-causing mutations (Table S1), suggesting that the c.6527insC mutation in particular shows this pattern. As expected, the main difference between New and Old-World individuals was a high prevalence of Native American segments in the New World, but also higher Iberian ancestry. No other Sephardic spikes of ancestry were observed elsewhere in the genome (Fig. 2b). This is consistent with our two-step model of Sephardic heritage for this mutation (Fig. 1b).

Pairwise identical-by-descent matching

Out of 8,128 possible pairs of matching participants ($128 \times 127/2$), 1,203 pairs (15%) were found to have matching segments after applying a 5 cM minimum IBD threshold (Fig. 3). Given the potentially long time elapsed since the cohort shared a common ancestor, and the wide variance of IBD overlap after just a few generations, many null matches were expected. Out of 15% of matching pairs, 1,035 of 1,203 (86%) matched at a segment spanning the c.6527insC mutation. We performed a one-sample Wilcoxon ranked-sign test to determine that 1,035 matches spanning c.6527insC is significantly higher than the genome-wide mean of 30 or 3% ($W = 0, p < 0.005, n = 100$). The Chilean samples showed the highest incidence of pairwise matching, probably owing to higher post-Columbian shared ancestry and/or endogamy. Notably, the two included Belmonte Sephardic samples (with no mutation; last two columns of Fig. 3) matched many mutation-carrying samples (with no previously known Sephardic ancestry).

Triangulation

We found a general pattern of autosomal clustering within countries of origin for most study participants (Fig. 4). Proximate countries in the New World (Argentina, Chile, and Colombia) and Old World (Spain, Portugal, France) were found on many adjacent branches of the hierarchical tree, suggesting shared post-Columbian ancestry between geographically close individuals. Interestingly, one French individual was found nested in a clade of Chilean individuals (BP52019) whereas 1 Chilean (MK40783) and 2 Argentinean samples were found nested in Spanish clades (MK65370 and MK65374). Most New World (particularly Chilean) clades were much more recently related than clades in other countries, consistent with our IBD results.

Age estimates for c.6527insC-containing haplotypes

Both Gamma and Alder methods of age estimation supported a pre-Columbian introduction of c.6527insC-containing haplotypes into Iberia (T_1), and subsequent post-Columbian introduction by this admixed population into the Americas (T_2 ; Table 2). Given the evidence from IBD and triangulation analysis of recent shared ancestry in the Americas, we favor the “correlated” genealogy approach of the Gamma method, however we report “independent” values for completeness. Generation times have varied considerably between eras and cultures, and we report several calendar dates based on generation times of 20, 25, and 30 years per generation. However, here we assume 30 years, because recent estimates suggest a value between 26–30.¹⁰

The Gamma method estimated T_1 to occur in 990 (95% CI: 371–1610) CE, whereas Alder estimated an earlier time of 767 (95% CI: 308–1226) CE. Although there is a 200-year difference in these mean estimates, their 95% confidence intervals are highly overlapping (Fig. S2), and both estimates coincide with the so-called Golden Age of Jews in Spain. As noted in the methods, the Gamma method estimates the TMRCA of an IBD segment, whereas Alder estimates the potentially

later time of population admixture. Convergent estimates by these two approaches likely implies a small donor population size.

The Gamma method estimated T_2 to occur in 1702 (95% CI: 1539–1859) CE, whereas Alder estimated an earlier time of 1665 (95% CI: 1650–1679) CE. These estimates are more consistent and have tighter confidence intervals, but are noticeably later than the post-Columbian era beginning in 1492 CE. This leaves the possibility that Jews carrying the c.6527insC mutation in Europe first assimilated there before migrating to the Americas generations later. This is consistent with our hypothesis (Fig 1b) and supported by the fact that more European than Native American segments flank the c.6527insC segment (Fig. 2a). A one-tailed binomial test confirmed that flanking segments to the mutation were more European than expected by chance ($p=2.62 \times 10^{-12}$).

Discussion

Genetics, culture, history and religion have unified the Jewish people since their origins in the Middle East more than 5,000 years ago.¹¹ Endogamy historically predominated in Jewish populations, giving rise to a high prevalence of genetic diseases. Previously unknown as prevalent in Jewish populations, RDEB was only recently recognized to have an association with Sephardic communities.⁶ The RDEB population in this study from Spain, France, Argentina, Chile, Colombia, and the USA, previously unknown to have Jewish origins, unambiguously demonstrate considerable Sephardic ancestry at the region surrounding the c.6527insC mutation (Fig. 2a & 2b). An unequivocal Sephardic lineage in this population can be explained by shared ancestors who underwent forced conversions to Catholicism on the Iberian Peninsula during the Spanish Inquisition. Crypto-Jewish communities emerged at this time of massive persecution so families could secretly maintain their Jewish faith while outwardly professing adherence to Catholicism. Descendants of crypto-Jews frequently lost their Jewish identity over time, however genetic diseases present in these communities consistently survived. The c.6527insC mutation undoubtedly has Jewish origins, arriving from the Middle East ~900 CE (767–990 CE; Table 2) during the “golden age”, when Jewish life was flourishing on the Iberian Peninsula (Fig. 1b). Thus, our results are broadly consistent with our hypothesized time course (Fig. 1b). The common ancestor(s) of RDEB individuals with the c.6527insC mutation dates to 1665–1702 CE (Table 2), during a post-Columbian era that saw the emergence of many crypto-Jewish communities.

The Spanish Inquisition is among the fiercest examples of a system enforcing religious intolerance and propagating genetic homogeneity, with consequences clearly still relevant to our world today.¹² It is estimated that persecuted Jewish individuals during the Inquisition represented approximately one-third of early Spanish immigrants and the propagation of a rare genetic disease, such as RDEB, in Iberian exile communities can be attributed, at least in part, to a founder effect.¹³

¹⁴ To date, there is scarce knowledge about Sephardic crypto-Jewish descendants, due to limited data from the original source of Sephardic Jews on the Iberian Peninsula combined with the challenge of identifying the existent communities today.¹⁵ Communities in Portugal (Belmonte and Bragança) and Mallorca (Chueta) have known crypto-Jewish ancestry. Interestingly, individuals from the Belmonte community were shown to distantly match RDEB individuals carrying the c.6527insC mutation, substantiating crypto-Jewish ancestry (Fig. 3 & 4).

The migration patterns of RDEB individuals with the c.6527insC mutation notably resemble the Sephardic Diaspora map after 1492 CE (Fig. 1a). While Sephardic ancestry predominates overwhelmingly at the region surrounding the c.6527insC mutation in this RDEB population, traces of Ashkenazi ancestry are also evident (Fig. 2a). The combination of both Sephardic and Ashkenazi ancestry at this region, suggests that the mutation predates Sephardic and Ashkenazi admixture during the Middle Ages.⁸ Jewish people have lived on the Iberian Peninsula as far back as the Roman Empire and the c.6527insC mutation can be traced back even further to pre-Roman communities on the Iberian Peninsula.¹⁶

Waves of Jewish people, including RDEB ancestors, migrated to the Americas from Europe after 1492 CE and greatly shaped the modern day Latin American population structure.¹³ The European, Native American and Sephardic admixture represented by the c.6527insC mutation reflects the migration of Jewish-Iberian people escaping the Iberian Peninsula during the Inquisition.^{14, 17} While many individuals did leave for the Americas, the majority in this RDEB subpopulation are Spanish, indicating a strong example of Jewish families remaining in Spain throughout the duration of the Inquisition. Many of those individuals who did leave Spain, settled in remote areas of Europe and the Americas where they were less likely to be discovered and exposed. A number of mutations were brought to the Americas via these routes, including the pathogenic growth hormone receptor (GHR) mutation in Laron syndrome, observed in the isolated Lojano community in Ecuador, a population known to have the influence of Sephardic crypto-Jewish ancestry.¹⁴

One particular region in North America well-known to attract immigrants who wished to maintain their Jewish practices under less scrutiny in the New World while demonstrating a veneer of Catholicism was the isolated San Luis Valley in northern New Mexico and southern Colorado.¹⁸ This region of the USA supports a population structure with greater frequencies of genetic diseases well-known in Jewish populations, including Pemphigus Vulgaris, Bloom Syndrome and BRCA1/BRCA2 associated breast cancer, suggesting those fleeing the Inquisition may have brought Jewish founder mutations to contemporary Hispanic populations.^{17, 19} The 185delAG mutation in BRCA1 was identified at a surprisingly high frequency in non-Jewish Colorado families originating in the San Luis Valley and similar to the RDEB population, were found to have Jewish ancestry.^{19, 20}

Interestingly, one of the RDEB individuals is heterozygous for c.6527insC and c.7485+5G>A, a mutation predominantly found in the San Luis Valley, supporting multiple RDEB mutations potentially integrated in crypto-Jewish communities. Further studies will elucidate the extent of RDEB mutations associated with Jewish ancestry. The association of rare diseases such as RDEB with a unique population like the one found in the San Luis Valley provides invaluable information to better serve isolated communities with rare diseases. Increased knowledge of the survival of crypto-Jewry and the genetic architecture of the Sephardic Jewish population will illuminate patterns of genetic diseases, such as RDEB. Furthermore, recognition of the RDEB subpopulation unified by the single and prevalent c.6527insC mutation, will enhance the efficient implementation of promising new RDEB therapies that have been accomplished in preclinical settings, including the *ex vivo* and *in vivo* correction of c.6527insC by multiple gene editing strategies.²¹⁻²⁶

Materials and Methods

Participant selection and sample collection of individuals with the c.6527insC mutation

A total of 132 RDEB homozygous and compound heterozygous patient samples from Spain, France, Argentina, Chile, Colombia and the United States with the c.6527insC mutation in *COL7A1* were used in this study. Their genotypes were previously identified using various sequencing technologies and subsequently confirmed by Sanger sequencing (Table S1).^{5, 16, 27, 28} Out of these, 126 were used in the final analysis (see “Autosomal genotyping and kinship estimation” below). Informed written consent was obtained from all patients in concordance with Institutional Review Board approval from Spain: UC3M Ethics Committee (Approval number: CEI21_15) France: Necker Comité de Protection des Personnes (Clinical Trials Reference number: NCT01874769) Argentina: Ethics Committee of the Ricardo Gutierrez Children's Hospital of Buenos Aires (Registry number: 16.38) Chile: Comité Etico Cientifico, Facultad de Medicina, Clinica Alemana—Universidad del Desarrollo (Project number: 2013-145) USA: Colorado Multiple Institutional Review Board (COMIRB no: 09-0192), and Colombia: Universidad del Rosario (CIE-UR DVO005 1149-CV1192). In addition, five Sephardic individuals (two kept in the final analysis) from the endogamous community of Belmonte, Portugal were included to evaluate the genetic relationship of RDEB-carrying individuals with a uniquely preserved crypto-Jewish community with thriving modern Sephardic people.

Autosomal genotyping and kinship estimation

Autosomal genotyping was performed by Gene by Gene, Ltd., on a customized version of the Infinium Global Screening Array-24 v3.0 BeadChip, and analyzed with the Family Finder autosomal DNA test.²⁹ This array includes approximately 700,000 SNPs for the 22 pairs of autosomal chromosomes and chromosome X. We used KING v2.2.4³⁰ to estimate kinship coefficient between each pair of 137 samples initially considered in the study. Individuals were removed from further analysis if they were third degree relatives or closer, resulting in a final total of 126 c.6527insC-carriers and two Belmonte individuals.

Local ancestry analysis

Local population ancestry was estimated for the 126 unrelated individuals carrying one or more copies of the c.6527insC mutation. Samples were first imputed to the Illumina Human OmniExpress BeadChip array SNP set for compatibility with all reference data. We used IMPUTE2³¹ and the 1000 Genomes³² reference panel with a union of SNPs from both chip types, and an imputation probability threshold of 0.9. Next, we used Eagle v2.4.1³³ for long-range phasing of haplotypes, and to impute any remaining no-calls. For a reference panel, we used an extensive collection (104,521) of FamilyTreeDNA (hereafter “FTDNA”) customers spanning each major worldwide population. With a genotyping rate of 0.988 and all SNPs present in the phasing

panel, we phased and imputed 637,645 SNPs across chromosomes 1 through 22. One positional Burrows–Wheeler transform (PBWT) phasing iteration using the default auto-selection process.

A reference panel for local ancestry classification was constructed to represent population ancestries from each major continent, as well as various Jewish and non-Jewish populations in Europe. We included nine reference populations in total: Sub-Saharan African, Native American, East Asian, South Asian, North European, South European, Ashkenazi Jewish, Sephardic Jewish from Turkey, and Sephardic Jewish from Morocco (Table 1).^{32, 34-37} Sample size disparities can adversely influence machine learning accuracy, so we randomly chose an approximately equal number of samples from each population. The two smallest sample sizes available were Turkish Sephardic (n=53) and Moroccan Sephardic (n=38), and inordinately small sample sizes can also be problematic. Therefore, we compromised on a sample size of 53 for all populations and used all available samples of Moroccan Sephardic.

We used a method similar to RFmix³⁸ for local classification but adapted by FTDNA for their proprietary tool myOrigins v3.0.³⁷ This method for classifying phased segments into populations was found to be more accurate than RFmix, and the subsequent step of smoothing out classification errors with a conditional random field is identical to that described in RFmix. We classified each 500 SNP segment, using a sliding window to move each overlapping segment across each chromosome in increments of 200 SNPs. A hidden Markov model (HMM) was used to correct switch errors implicit in imperfect phasing, by predicting the true (hidden) diploid phase of each maternal and paternal segment, given the observed order of population labels.³⁷ The transition probabilities used were as follows: no strand flip (0.850; e.g., 1/2 to 1/2), strand flip (0.000; e.g., 1/2 to 2/1), partial overlap (0.128; e.g., 1/2 to 1/3), partial overlap and strand flip (0.017; e.g., 1/2 to 3/1), other (0.005; e.g., 1/2 to 3/4). Results were processed and plotted using custom scripts with R v4.1.2.³⁹

Pairwise identical-by-descent matching

We used the Family Finder algorithm to perform a SNP-wise comparison for each pair of all 128 individuals to find shared segments, defined as runs of SNPs sharing at least one allele. Seed segments were formed from at least 900 adjacent matching SNPs, and extended in both directions, to merge with adjacent segments. Unqualified segments were then discarded, if they did not contain at least 480 SNPs, a genetic distance of 2 centimorgans (cM), or a density of 105 SNPs/cM. We used the linkage map (build 37) from the 1000 Genomes Project.³² Given the potentially very distant identical-by-descent (IBD) matches expected amongst descendants of a centuries-old Jewish diaspora, we used custom filters to determine whether two individuals “match.” A match is defined as two people with at least 5 cM in common and allowing for shared segments ≥ 2 cM. We summarized pairwise matches overall, and those with segments overlapping the c.6527insC mutation, in R v4.1.2.³⁹

Triangulation

We used triangulation as an additional validation to more conservatively assess IBD matching between all 128 samples. Triangulation⁴⁰ is the transitive property of shared DNA: segments that are shared IBD between three or more individuals from a recent common ancestor should match pairwise between all three, at an overlapping genetic location. Any segments with a total overlap between group members of < 2 cM were removed. We constructed a dendrogram based on a hierarchical structure analysis of total shared triangulated DNA to find clusters of more closely related individuals. This assumes all individuals have recent common ancestry through just one genealogical line, through which they inherited the c.6527insC mutation. We used the `hclust` and `dendrogram` functions in base R, with the complete linkage method to find similar clusters.³⁹ Distances were computed as $1 - (cM/cM_{max})$.

Age estimates for c.6527insC-containing haplotypes

We used two independent methods to estimate the date that c.6527insC was introduced, first from Sephardic people into Iberia, and later into the Americas. First, we applied the “Gamma” method.^{41, 42} The basic theory is that, under the Haldane recombination model,⁴³ the length between recombination breakpoints is a random Poisson process, and exponentially distributed with rate of 1.0. This simple model does not account for interference (inhibition of one crossover by another) so is not perfectly accurate. However, at shorter genetic distances, the likelihood (and effect) of multiple recombination events is negligible. The logic is as follows: a mutation is inherited within a haplotype, which is iteratively broken into smaller segments with each successive meiosis. The mean distance from mutation to one of its two flanking recombination breakpoints is $1/\tau$ Morgans, or $100/\tau$ cM, where τ is the time to most recent common ancestor (TMRCA) in units of generations. Note: the TMRCA for a group of mutation-carriers may be (substantially) younger than the TMRCA of the mutation itself, since it only considers a small subset of all mutation-carriers, past and present. Therefore, the length of the entire segment is distributed as *Gamma*(2, τ), with expected length $2/\tau$ Morgans, or $200/\tau$ cM.

The method of Gandolfo⁴² corrects for the bias of small sample size of segment lengths l_i , with a bias correction factor, $b(n) = (2n - 1)/2n$. Using this correction, they estimate the TMRCA as:

$$\hat{\tau} = \frac{b(n)2n}{\sum_{i=1}^n l_i}$$

Exact confidence intervals are then calculated from $\tau/\hat{\tau}$. This calculation assumes that all mutation-carriers are unrelated (“independent”) since their TMRCA, i.e., no close cousin relationships, which would break the distributional assumption of independent and identically distributed samples. A second “correlated” version of the calculation accounts for the possibility of reticulating history, using the bias correction factor $b(n*) = 2n/(1 + (n - 1)\rho)$, where ρ is the mean pairwise correlation between segment lengths, and $\bar{l}$ and s^2 are respectively the mean and variance of segment lengths:

$$\hat{\rho} = \frac{n\bar{l} - s^2(2n + 1)}{n\bar{l} + s^2(n - 1)}$$

For the Sephardic-Spanish time estimate (T_1), we used the population segment edges derived from myOrigins v3.0. Segment boundaries were defined as start and end points of a Sephardic segment spanning the mutation at position 48,611,297 on chr3.⁴⁴ For compound heterozygotes, we only used the haplotype carrying the c.6527insC mutation. This haplotype was determined by IBD matching to c.6527insC-homozygous individuals and selecting the best match (i.e., having the fewest nucleotide mismatches). For the American time estimate (T_2), we used the maximum of pairwise IBD matching segments derived from Family Finder. Only segments from New World individuals were used for T_2 .

The second age estimate method we applied was Alder v1.03,⁴⁵ which uses patterns of linkage disequilibrium (LD) decay to compute time since an admixture event. Alder builds upon previous methods^{46, 47} which show that admixture LD scales with time since admixture (t), genetic distance (d), and the initial difference (D_0) in allele frequencies between mixing populations:

$$D_t \approx e^{-td} D_0$$

The method fits a least-squares curve to patterns of exponential LD decay, to solve for the number of generations since admixture, and then calculates confidence intervals by jackknifing each chromosome.

We used Alder to estimate T_1 as the admixture date between Sephardic and Iberian references, with Old World RDEB individuals as the admixed population. We then estimated T_2 as the admixture date between Native American references and Old World RDEB individuals, with New World RDEB individuals as the admixed population. For both estimations, we considered a range of genetic distances between 0.05 and 50 cM. We converted g generations with generation time gt to years before present as $gt \times (g + 1)$, to account for the unknown age of participants.

Importantly, each of the events we tested (T_1 and T_2) actually consists of two potentially different time periods: (a) the TMRCA of an IBD segment, and (b) the potentially later time of population admixture. For example (T_2), the ancestral population of Sephardic-Iberian mutation donors could have lived in Europe for some time prior to colonization of the Americas, and subsequent intermixing with Native Americans. Gamma gets at (a) whereas Alder gets at (b). However, (a) and (b) should converge on the same time period if the population size of donors was small. Although we hereafter refer to T_1 and T_2 as each being a single time period, we note the possibility that Gamma could overestimate the admixture time compared to Alder.

Table 1. Reference populations used for local ancestry classification, and their sources.

Target Population*	Proxy Population†	N‡	Citation§
Sub-Saharan African	Nigerian	53	Siva 2008
Native American	Native American groups in South America: Cachi, Colla, Quechua, unknown tribal affiliation in Ecuador, Peru, and Bolivia	53	Eichstaedt et al. 2014; Pagani et al. 2016; Mallick et al. 2016; Maier et al. 2021
East Asian	South Han Chinese	53	Maier et al. 2021
South Asian	Dravidian	53	Siva 2008
North European	British	53	Maier et al. 2021
South European	Iberian	53	Maier et al. 2021
Ashkenazi Jewish	Ashkenazi Jewish	53	Maier et al. 2021
Sephardic Jewish in Turkey	Sephardic Jewish in Turkey	53	Maier et al. 2021
Sephardic Jewish in Morocco	Sephardic Jewish in Morocco	38	Maier et al. 2021

* Continental or regional population of interest

† Specific reference population used in local ancestry analysis

‡ Sample size; all non-Jewish reference populations were capped to the largest Jewish sample size

§ Study from which reference samples were drawn

Table 2. Age estimates for admixture of c.6527insC mutation into Iberia and Americas.

Admixture Event*	Meth od†	Assumption‡	Generatio ns BP§	Calendar Date (20 Y/G)¶	Calendar Date (25 Y/G)¶	Calendar Date (30 Y/G)¶
Sephardic + Spanish	Gam ma	'Independent' genealogy	29.7 (26.4-33.5)	1408 (1333–1475) CE	1254 (1160–1338) CE	1101 (988–1201) CE
Sephardic + Spanish	Gam ma	'Correlated' genealogy	33.4 (12.8-54.1)	1334 (922–1748) CE	1162 (647–1679) CE	990 (371–1610) CE
Sephardic + Spanish	Alder	-	40.9 (25.6-56.2)	1186 (880–1492) CE	976 (594–1359) CE	767 (308–1226) CE
American + Span-Seph	Gam ma	'Independent' genealogy	8.9 (7.5-10.6)	1824 (1790–1853) CE	1774 (1732–1810) CE	1725 (1674–1767) CE
American + Span-Seph	Gam ma	'Correlated' genealogy	9.7 (4.5-15.1)	1809 (1701–1914) CE	1755 (1620–1886) CE	1702 (1539–1859) CE
American + Span-Seph	Alder	-	10.9 (10.4-11.4)	1784 (1774–1794) CE	1725 (1712–1737) CE	1665 (1650–1679) CE

* Which modeled admixture event, either T₁ or T₂

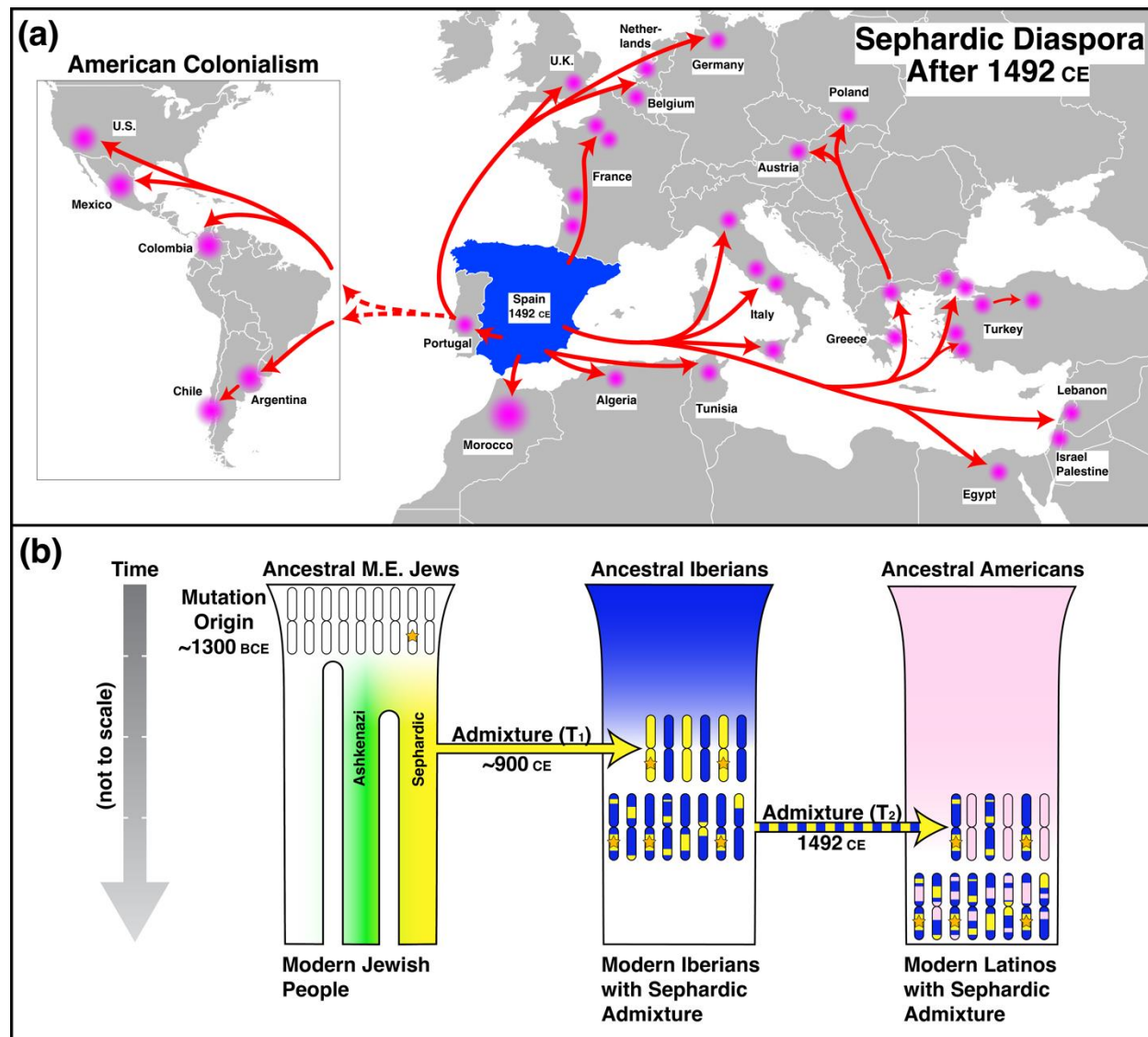
† Age estimation method

‡ Assumption of Gamma method

§ Mean estimated age in generations before present, with 95% CI

¶ Mean estimated age in calendar year (see assumed generation time), with 95% CI

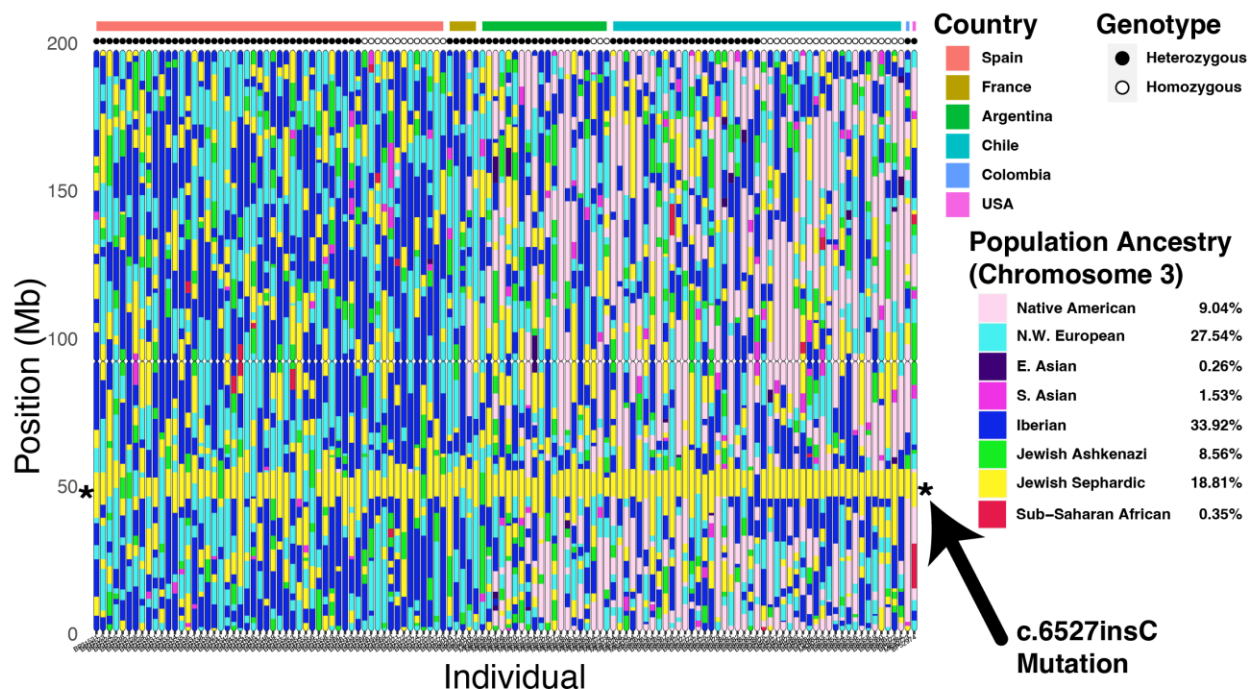
Figure 1. Sephardic Diaspora and the c.6527insC Mutation



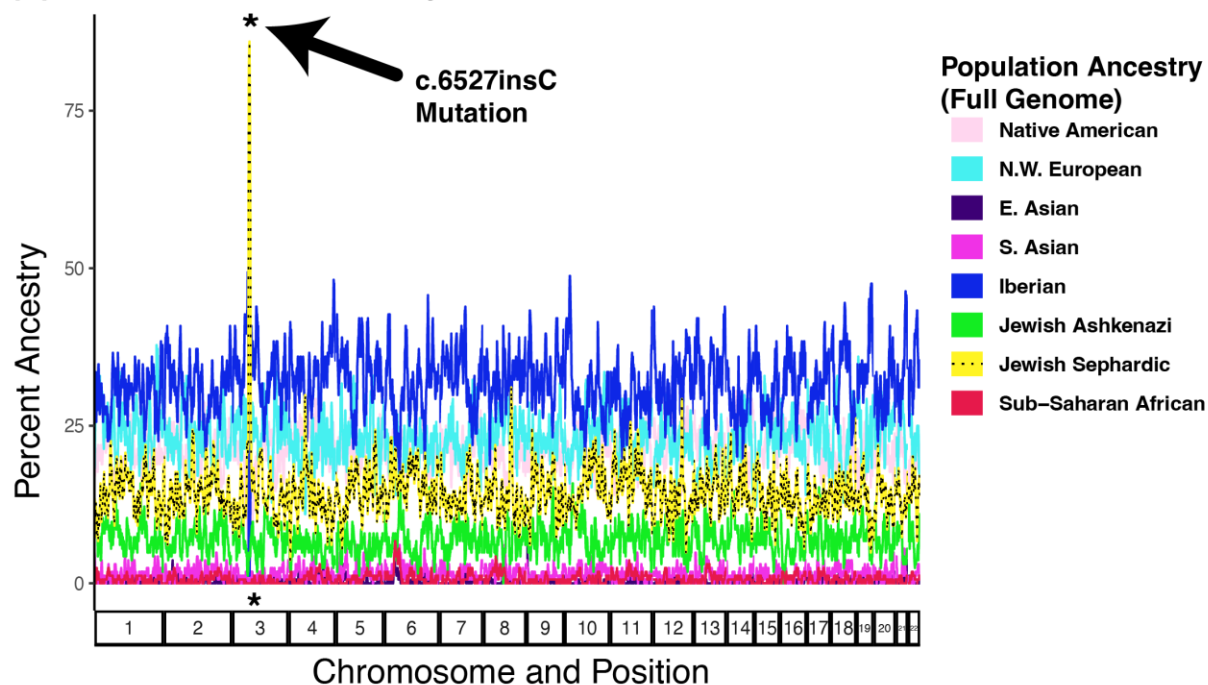
(a) Geographic representation of Sephardic Diaspora during post-Columbian history (after 1492 CE). Routes and locations are only approximate and likely incomplete. (b) Conceptual historical model of the c.6527insC mutation, with hypothesized times to be tested. First (T₀), the mutation arose in a single ancestor during the Iron Age.¹⁶ Next (T₁), a group of Sephardic individuals with high prevalence of the mutation migrated to Iberia where they admixed with local Spaniards. This may have occurred during the Golden Age of Jews in Spain.^{6, 16} Finally (T₂), many post-Columbian diaspora ensued following the Spanish Inquisition, including converso Sephardic individuals who migrated to the Americas. Importantly, this model predicts the mutation to be overlapped by primarily Sephardic DNA segments in both Old and New World individuals.

Figure 2. Sephardic Local Ancestry Spans c.6527insC Mutation

(a) Chromosome Painting for 126 Individuals (Haploid)

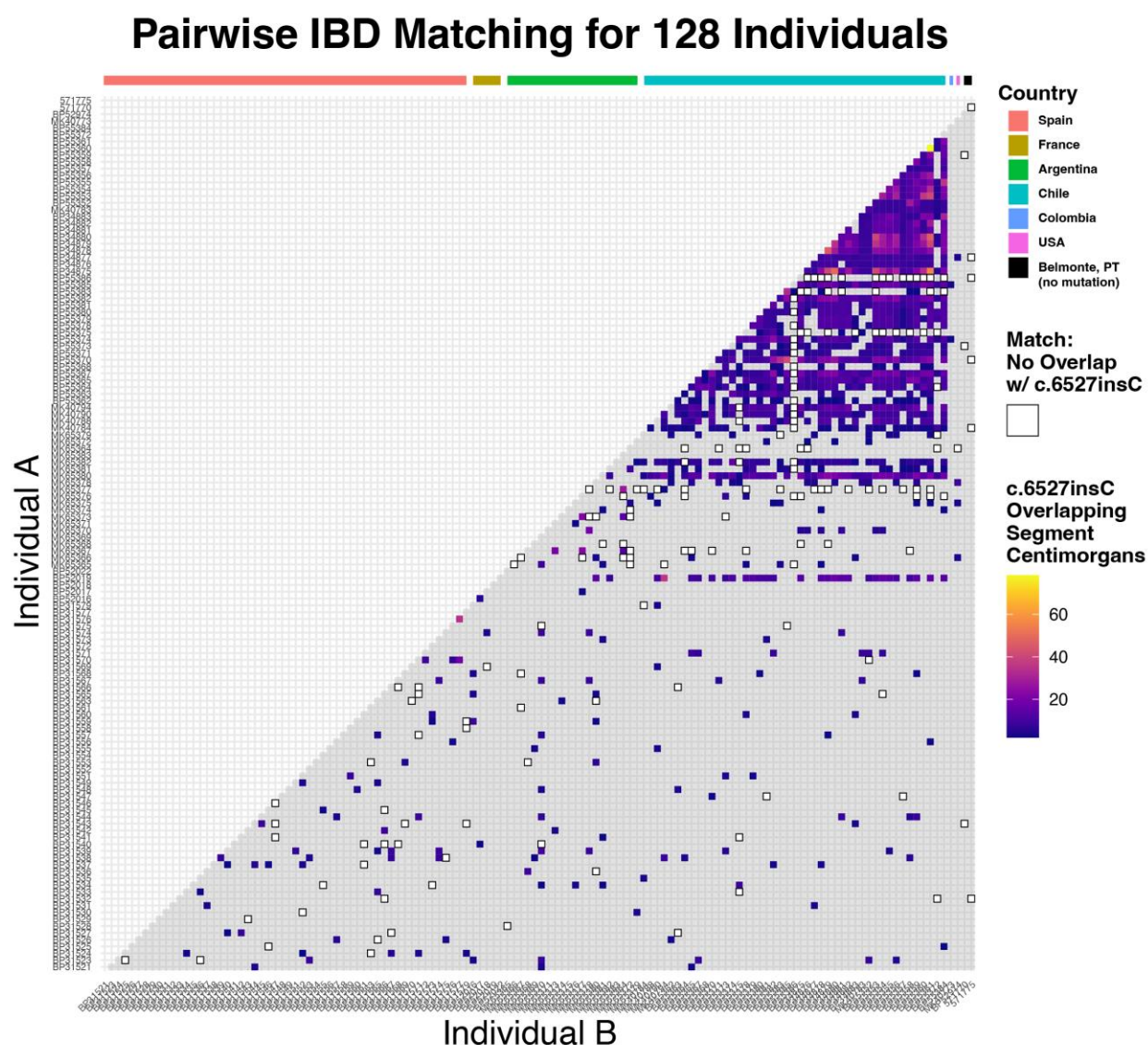


(b) Percent Ancestry at Each Genomic Position



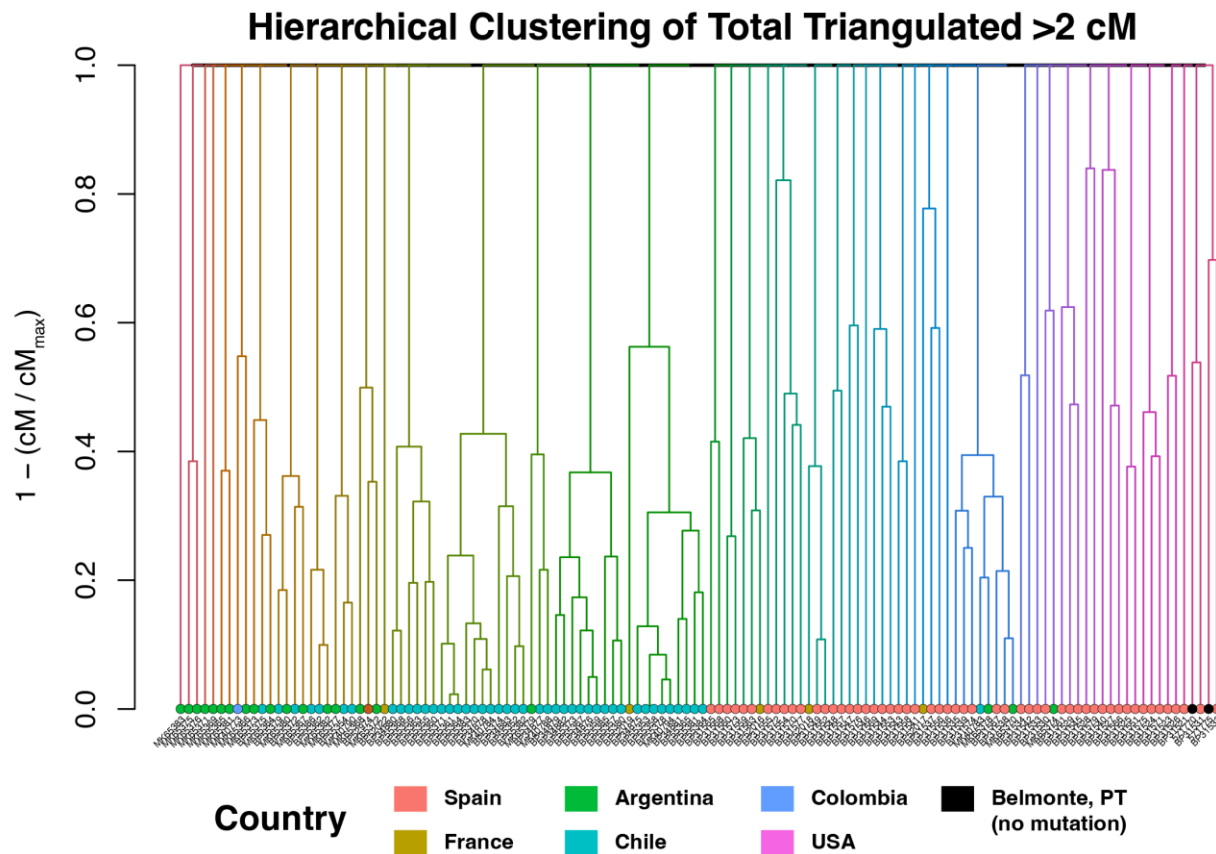
(a) Results of myOrigins v3.0 analysis showing local and overall population ancestry for chromosome 3. All 126 unrelated carriers of c.6527insC mutation are shown for the haplotype that is most Sephardic at position 48,611,297. Turkish and Moroccan Sephardic ancestry are merged into one group for display due to lower sample sizes and genetic similarity. Country of origin is denoted by rainbow colors, and genotype is denoted by circles. (b) Genome-wide population ancestry aggregated across all 126 samples. Both haplotypes are included for homozygotes, and one haplotype (best match to homozygotes) is included for heterozygotes. A peak of 83% Sephardic ancestry is found at chr3, position 48,611,297.

Figure 3. Pairwise IBD Matching



Family Finder pairwise matching with relevant thresholds applied between all pairs of 128 samples (including two Belmonte samples). Any IBD match spanning the c.6527insC mutation is shown by plasma colors denoting the segment length in cM. If a pair matches but without spanning the mutation, an empty box is shown. Rainbow colors denoting country of origin are identical to those in Fig. 2, except that Belmonte samples are shown in black.

Figure 4. Autosomal Relationships of Study Participants



Hierarchical clustering tree showing proximity of the relationship for all 128 individuals in the study. Clustering was performed using total cM for all triangulated DNA segments, i.e., those matching between three or more individuals. Vertical axis is normalized between zero and one. Rainbow colors for circles denote country of origin and are identical to those in Fig. 3. Rainbow colors for tree branches denote clusters.

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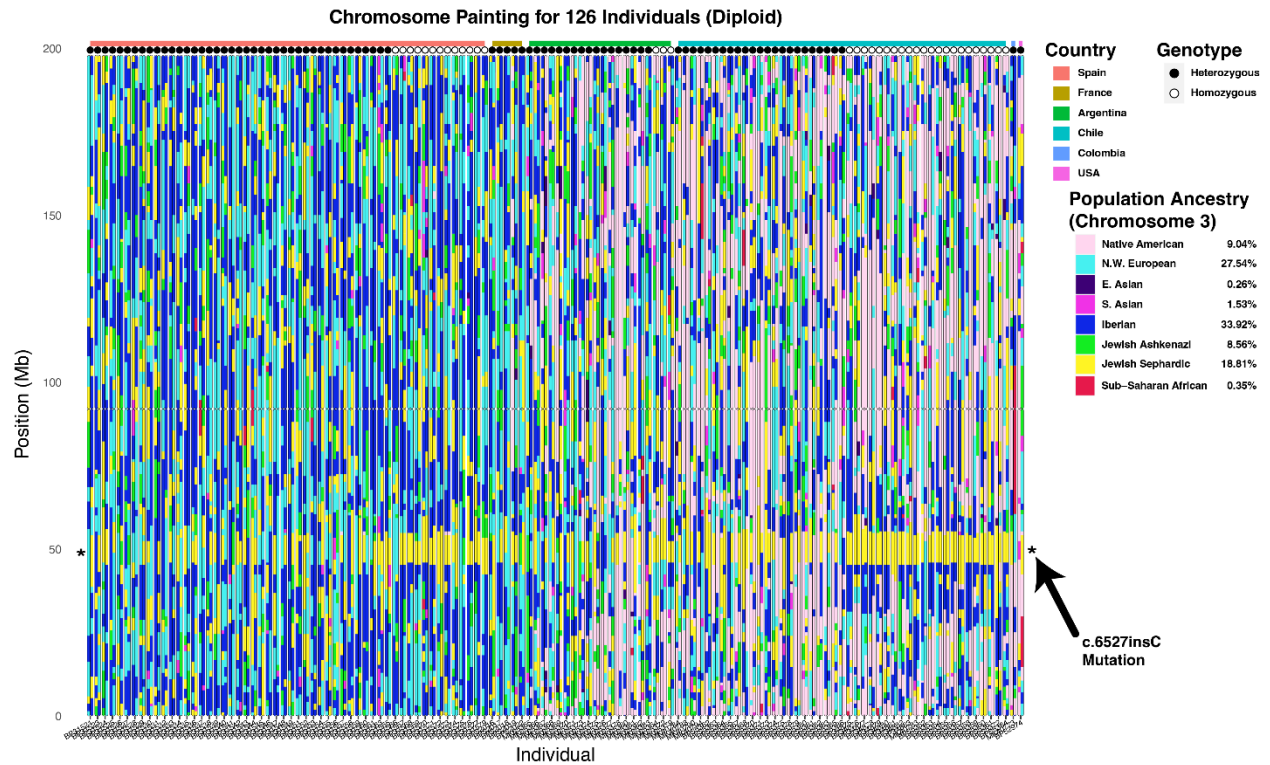
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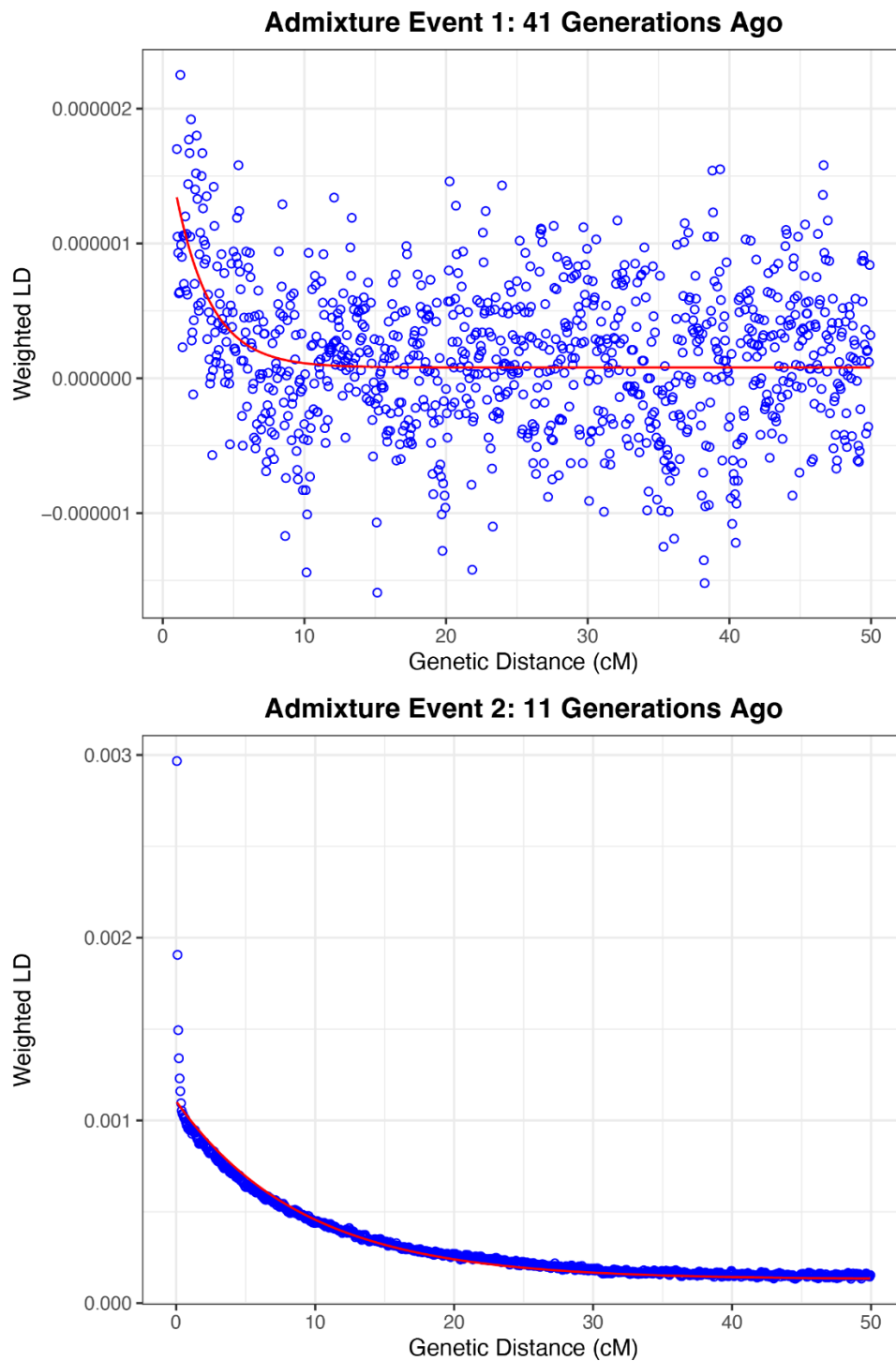
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Figure S1. Sephardic Local Ancestry Spans c.6527insC Mutation



Results of myOrigins v3.0 analysis showing local and overall population ancestry for chromosome 3. All 126 unrelated carriers of c.6527insC mutation are shown for both haplotypes, including the haplotype that is most Sephardic at position 48,611,297. Turkish and Moroccan Sephardic ancestry are merged into one group for display due to lower sample sizes and genetic similarity. Country of origin is denoted by rainbow colors, and genotype is denoted by circles.

Figure S2. TMRCA Estimates from Alder Analysis



Linkage disequilibrium (LD) decay curves from Alder Analysis of the two admixture events depicted in Figure 1: T_1 (top; 41 generations ago) and T_2 (bottom; 11 generations ago). The amplitude of the exponential decay curve can be translated into generations before present.