A Human Stem Cell-Based System to Study the Role of TP63 Mutations in Ectodermal Dysplasias


TO THE EDITOR
Point mutations in the transcription factor TP63 gene underlie a subset of ectodermal dysplasias, including ankyloblepharon-ectodermal defects–cleft lip/palate syndrome (AEC) (OMIM 106260), a developmental disorder associated with severe skin erosions (McGrath et al., 2001) (Figure 1a). TP63-AEC mutations cluster in exons 13 and 14, exons that encode putative protein-protein interaction domains (Figure 1b). Molecular mechanisms causing skin erosions in AEC are poorly understood. Both mouse models (Ferone et al., 2013; Koster et al., 2009; Russo et al., 2018) and human keratinocyte models (Zarnegar et al., 2012) have been used to investigate aspects of AEC. Although both approaches yielded important results, they also suffered from significant drawbacks. The mouse models did not fully replicate AEC skin phenotypes, and the cell culture models used nonphysiological overexpression of mutant TP63-AEC in primary human keratinocytes (see discussion in Koch et al., 2014). In this study, we used AEC patient-derived cells that express physiological levels of TP63-AEC alleles on a genetic background susceptible to the disease. The latter point is important because disease severity among patients carrying the same TP63 mutation can vary dramatically (e.g., Bertola et al., 2004).

Initially, we generated fibroblast-derived induced pluripotent stem cells (iPSCs) from two AEC patients carrying mutations in either exon 13 (I537T [line F5]) or exon 14 (R598L [line E3]) of the TP63 gene (Figures 1b and c). Biopsy samples were obtained with institutional approval and written informed parental consent. Next, we used CRISPR/CAS- and TALEN-mediated gene editing to correct the TP63-AEC mutations in both lines (see Supplementary Figure S1 online). This yielded consisogenic pairs of iPSC lines that were genetically identical except for the presence or absence of the TP63-AEC mutation (E3, E3 gene corrected [GC]; F5, F5GC). These cell pairs are ideally suited for investigating

Figure 1. Generating iPSCs and iPSC-derived keratinocytes from AEC patient skin. (a) Patient affected by AEC exhibiting severe skin erosions. The patient and his parents consented to the use of this image in this publication. (b) TP63 mutations in AEC patients occur mainly in exons 13 and 14, encoding putative protein-protein interaction domains. Approximate location of mutations in the AEC patient cells (F5 and E3) used in this article are indicated by stars. The protein schematic shown is of ΔNp63α, the predominantly expressed TP63 isoform in human keratinocytes. Phase contrast images of (c) human iPSC colony and (d) iPSC-derived keratinocytes. (e) Disease pathways associated with the TP63 mutations in E3 and F5 iPSC-derived keratinocytes as determined by a Human Phenotype Ontology analysis of our RNA-sequencing data. AEC, ankyloblepharon-ectodermal defects–cleft lip/palate syndrome; DBD, DNA binding domain; FDR, false discovery rate; iPSC, induced pluripotent stem cell; OD, oligomerization domain; PS, post-SAM domain; SAM, sterile alpha motif.

Abbreviations: AEC, ankyloblepharon-ectodermal defects–cleft lip/palate syndrome; GC, gene corrected; iPSC, induced pluripotent stem cell; iPSC-K, induced pluripotent stem cell-derived keratinocyte

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molecular pathways while minimizing genetic background effects. The iPSCs were then differentiated into keratinocytes (Figure 1d) (Dinella et al., 2014). These iPSC-derived keratinocytes (iPSC-Ks) were similar to primary human keratinocytes in that they expressed keratinocyte markers (TP63 and KRT14) and assembled desmosomes containing DSG1/2, DSC3, DSG3, and JUP (Figure 2). Because TP63, KRT14, DSG3, and DSC3 are co-expressed only in stratified epithelia, these findings show that our iPSC-Ks show basic properties of keratinocytes. Next, we subjected the conisogenic iPSC-K lines, grown under conditions that suppress differentiation (low calcium culture conditions), to an RNA-sequencing analysis (accession number GSE109185). Upon analyzing
the ranked gene lists using the Human Phenotype Ontology tool (Kohler et al., 2017), we identified several abnormalities in the gene expression patterns of AEC iPSC-Ks that are relevant for the AEC skin phenotype (Figure 1e). Further analysis of the transcriptome data using Gene Ontology showed a significant effect of the AEC mutations on genes in the “Desmosome” category (FDR 1.49e-03). Specifically, we identified a down-regulation of desmosomal adhesins, a finding that we independently verified by semi-quantitative real-time reverse transcriptase–PCR (data not shown).

Next, we induced iPSC-K differentiation and desmosome assembly by exposing the cells to media with elevated calcium concentrations and performed immunofluorescence analysis for various desmosomal markers. We observed striking differences between AEC iPSC-Ks and gene-corrected iPSC-Ks in the expression level and distribution of several desmosomal transmembrane receptors (Figures 2b–d, F5 and F5GC cells are shown). To determine whether the abnormal expression and distribution of desmosomal proteins affected cell adhesion, we subjected these cells to a dispase cell adhesion assay (Hartlieb et al., 2014). In sharp contrast to gene-corrected iPSC-K sheets, AEC iPSC-Ks exhibited a disintegrated upon exposure to mechanical stress (Figure 2e). Enzyme-release assays suggested acantholysis (loss of cell–cell adhesion) rather than cytolsis as the underlying cause (data not shown).

By Western blotting, we determined that DSG3 and JUP were down-regulated in AEC iPSC-Ks (Figure 2g, and see Supplementary Figure S2 online). Down-regulation of these two proteins has been shown to activate p38 MAPK signaling and ultimately cause acantholysis in keratinocyte models of the autoimmune disease pemphigus vulgaris (Hartlieb et al., 2014; Spindler et al., 2014). To test whether aberrant DSG3-JUP-p38 MAPK signaling might contribute to acantholysis in AEC iPSC-Ks, we repeated the dispase assays in the presence of a p38 MAPK inhibitor (SB202190). The inhibitor stabilized the AEC iPSC-K sheets (Figure 2f), suggesting that activation of p38 MAPK signaling contributes to acantholysis. We have previously shown that JUP can act as a positive regulator of Dsc3 gene expression and that loss of Dsc3 or Dsg3 leads to acantholysis in mouse epidermis (Chen et al., 2008; Koch et al., 1997; Tokonzaba et al., 2013). This suggests that simultaneous down-regulation of all three proteins might exacerbate desmosomal adhesion defects in AEC iPSC-Ks.

We also observed reduced expression of cytoskeletal and desmosomal proteins associated with keratinocyte differentiation, including DSG1, DSC1, and KRT1 in AEC iPSC-Ks cultured in high calcium media (Figure 2g). A direct role for DSG1 in mediating keratinocyte differentiation through suppression of ERK signaling has been shown by Gtsios et al. (2009), who showed that knockdown of DSG1 in three-dimensional keratinocyte cultures leads to increased ERK signaling and reduced expression of the differentiation markers DSC1 and KRT1. Our Western blot data suggest that this mechanism also operates in AEC iPSC-Ks (Figure 2g). Thus, two previously identified signaling functions of desmosomes, DSG3-JUP-p38 MAPK–controlled cell adhesion and DSG1-ERK–controlled differentiation, are affected by the two different TP63-AEC mutations analyzed.

To determine whether our iPSC-based in vitro system phenocopies AEC epidermis, we next performed immunofluorescence staining of perilesional skin of AEC patients and showed focal loss (or reduced expression) of DSC3, DSG1, DSC1, and KRT1 (Figure 2h–m) (Koster et al., 2009), thereby validating the results obtained with our iPSC-based system. It remains to be seen why these desmosomal defects occur focally given that there is no evidence of mosaicism in AEC.

In summary, we have developed a human iPSC-based in vitro system that enables us to identify molecular mechanisms underlying skin fragility in AEC patients. Because iPSC-Ks from two patients with two different mutations showed similar desmosomal abnormalities, our data suggest that these defects are of general relevance for AEC. Our in vitro system will be ideally suited to identify new disease pathways in AEC and other ectodermal dysplasias caused by TP63 mutations.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
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REFERENCES
Dendritic cells (DCs) in the skin are critical for the development of adaptive immune responses to pathogens and for the maintenance of peripheral tolerance (Kashem et al., 2017). Under steady-state conditions, at least four subsets of cutaneous DCs have been characterized: epidermal Langerhans cells (LC), dermal classical DC1 (cDC1, also known as CD11b^+ DC), dermal cDC2 (also known as CD11b^+ DC), and CD103^-CD11b^- double-negative DCs. Although functional specializations of each DC subset have been well studied, the functional interaction between these DC subsets under steady-state conditions is poorly understood.

Targeting antigen to DC using monoclonal antibodies specific for C-type lectin receptors offers an efficient method to study adaptive responses to antigen presentation by targeted populations of DCs in vivo, and is being developed for therapeutic vaccinations (Steinman and Banchereau, 2007). We have previously developed BAC transgenic mice (huLang) with selective expression of human Langerin, a C-type lectin receptor, on epidermal LC (Bobr et al., 2010). Immunization of these mice with as little as 0.05 μg of α-huLangerin mAb conjugated to the 2W1S model antigen (α-huLang-2W1S) efficiently and selectively targeted LCs, resulting in expansion of endogenous 2W1S-specific CD4^+ T cells, which could be detected using 2W1S-I-A^b tetramer (Yao et al., 2015).

To examine whether LCs directly induce T-cell responses after antigen targeting, we crossed huLang mice with mice that were constitutively ablated of major histocompatibility complex (MHC) II selectively in LCs (huLang LC^ΔMHC II mice) (Igyarto et al., 2009). LCs in these mice express human Langerin, allowing for targeting with α-huLang-2W1S and should also lack expression of MHC II. To confirm the loss of MHC II, single-cell epidermal suspensions were isolated from flank skin and examined by flow cytometry. As expected, LCs gated as MHC II^+ were absent in huLang LC^ΔMHC II mice expressing levels of MHC II equivalent to MHC II^+/− mice (Figure 1a, 1b).

Next, huLang LC^ΔMHC II and huLang mice were injected intraperitoneally with 1 μg α-huLang-2W1S. As expected, endogenous 2W1S-I-A^b tetramer binding CD4^+ T cells isolated from lymph nodes and spleen 7 days later had expanded approximately 10-fold in huLang mice compared to WT lacking huLang expression (Figure 1c). Surprisingly, 2W1S-I-A^b-specific T cells expanded to a similar extent in huLang LC^ΔMHC II mice. To ensure that LCs in huLang LC^ΔMHC II mice did not retain levels of MHC II expression below the sensitivity of flow cytometry, we next FACS-sorted LCs from the epidermis and cultured them in vitro with OVA232–239 peptide and native carboxyfluorescein succinimidyl ester–labeled OT-II T cells. Sorting purity for this and all subsequent experiments was >95% (data not shown). After 4 days, robust dilution of carboxyfluorescein succinimidyl ester was observed in OT-II cells incubated with LCs isolated from wild-type mice, but not in cells incubated with LCs isolated from MHC II^+/− or huLang LC^ΔMHC II mice (Figure 1d). This was skin fragility in AEC syndrome [e-pub ahead of print]. Proc Natl Acad Sci USA 2018. https://doi.org/10.1073/pnas.1713773115 (accessed 9 March 2018).

