HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

J Invest Dermatol. 2018 July ; 138(7): 1662–1665. doi:10.1016/j.jid.2018.02.016.

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

Jason D. Dinella[1,2], **Jiangli Chen**[1,2], **Saiphone Webb**[1,2], **Elaine Siegfried**[3], **Alanna F. Bree**[4], **Senthilnath Lakshmanachetty**[1,2], **Velmurugan Balaiya**[1,2], **Maranke I. Koster**[1,2,6,*], and **Peter J. Koch**[1,2,5,6,*]

[1]Department of Dermatology, University of Colorado Medical School, Aurora, CO

[2]Gates Center for Regenerative Medicine, University of Colorado Medical School, Aurora, CO

[3]Department of Dermatology, St Louis University, St. Louis, MO

[4]Department of Dermatology, Baylor College of Medicine, Houston, TX

[5]Departments of Cell and Developmental Biology, University of Colorado School of Medicine

[6]Departments of Ophthalmology, University of Colorado School of Medicine

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, while the cell culture models used non-physiological overexpression of mutant TP63-AEC in primary human keratinocytes [see discussion in (Koch et al., 2014)]. In the present study, we utilized 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 as disease severity among patients carrying the same *TP63* mutation can vary dramatically (e.g. Bertola et al., 2004).

Corresponding author: Peter J. Koch, Ph.D., Department of Dermatology, 12800 East 19th Avenue, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, Phone: (303) 724-0051, Fax: (303) 724-3051, Peter.Koch@ucdenver.edu.
*PJK and MIK contributed equally to this work.

Conflict of Interest: The authors state no conflicts of interest

Initially, we generated fibroblast-derived induced pluripotent stem cells (iPSC) 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, c)(biopsies were obtained with institutional approval and written informed patient consent). Next, we used CRISPR/CAS- and TALEN-mediated gene editing to correct the *TP63*-AEC mutations in both lines (Supplementary Figure S1). This yielded conisogenic pairs of iPSC lines that were genetically identical except for the presence or absence of the *TP63*-AEC mutation [E3, E3GC (gene-corrected); F5, F5GC]. These cell pairs are ideally suited to investigate molecular pathways while minimizing genetic background effects. The iPSC were then differentiated into keratinocytes [Figure 1d and (Dinella et al., 2014)]. These iPSC-derived keratinocytes (iPSC-K) 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). As TP63, KRT14, DSG3, and DSC3 are only co-expressed in stratified epithelia, these findings demonstrate that our iPSC-K 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 RNAseq 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 gene expression patterns of AEC iPSC-K which are relevant for the AEC skin phenotype (Figure 1E). Further, analysis of the transcriptome data using Gene Ontology revealed a significant effect of the AEC mutations on genes in the “Desmosome” category (FDR 1.49e-03). Specifically, we identified a downregulation of desmosomal cadherins, a finding that we independently verified by qRT-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-K and gene-corrected iPSC-K 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-K sheets rapidly disintegrated upon exposure to mechanical stress (Figure 2e). Enzyme-release assays suggested acantholysis (loss of cell-cell adhesion) rather than cytolysis as the underlying cause (data not shown).

By Western blotting, we determined that DSG3 and JUP were downregulated in AEC iPSC-K (Figure 2g and Supplemental Figure 2). Downregulation of these two proteins has been shown to activate p38MAPK 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-p38MAPK signaling might contribute to acantholysis in AEC iPSC-K, we repeated the dispase assays in the presence of a p38MAPK inhibitor (SB202190). The inhibitor stabilized the AEC iPSC-K sheets (Figure 2f), suggesting that activation of p38MAPK signaling contributes to acantholysis. Interestingly, 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 downregulation of all three proteins might exacerbate desmosomal adhesion defects in AEC iPSC-K.

We also observed reduced expression of cytoskeletal and desmosomal proteins associated with keratinocyte differentiation, including DSG1, DSC1, and KRT1 in AEC iPSC-K cultured in high calcium media (Figure 2g). A direct role for DSG1 in mediating keratinocyte differentiation through suppression of ERK signaling has been demonstrated (Getsios et al., 2009). These authors showed that knockdown of DSG1 in 3D 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-K (Figure 2g). Thus, two previously identified signaling functions of desmosomes, DSG3-JUP-p38MAPK-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 demonstrated focal loss (or reduced expression) of DSC3, DSG1, DSC1, and KRT1 [Figure 2h-m; and (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. As iPSC-K from two patients with two different mutations demonstrated 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) of the National Institutes of Health (NIH) under Award Numbers R56 AR066713 and R01AR072621. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. Further support was provided by research grants from the National Foundation for Ectodermal Dysplasias (NFED). MIK and PJK were the recipients of both grant mechanisms. iPSC line generation and characterization were performed with support from the Gates Bioengineering Core at the University of Colorado Anschutz Medical Campus. Bioinformatics support was provided by Drs. Michael Edwards and Tzu Phang (University of Colorado). We would like to thank the patients who supported this research by providing skin biopsies.

Abbreviations

AEC	Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome
DSG	Desmoglein
DSC	Desmocollin

JUP	Plakoglobin
KRT	Keratin
iPSC	Induced Pluripotent Stem Cells
iPSC-K	iPSC-Derived Keratinocytes

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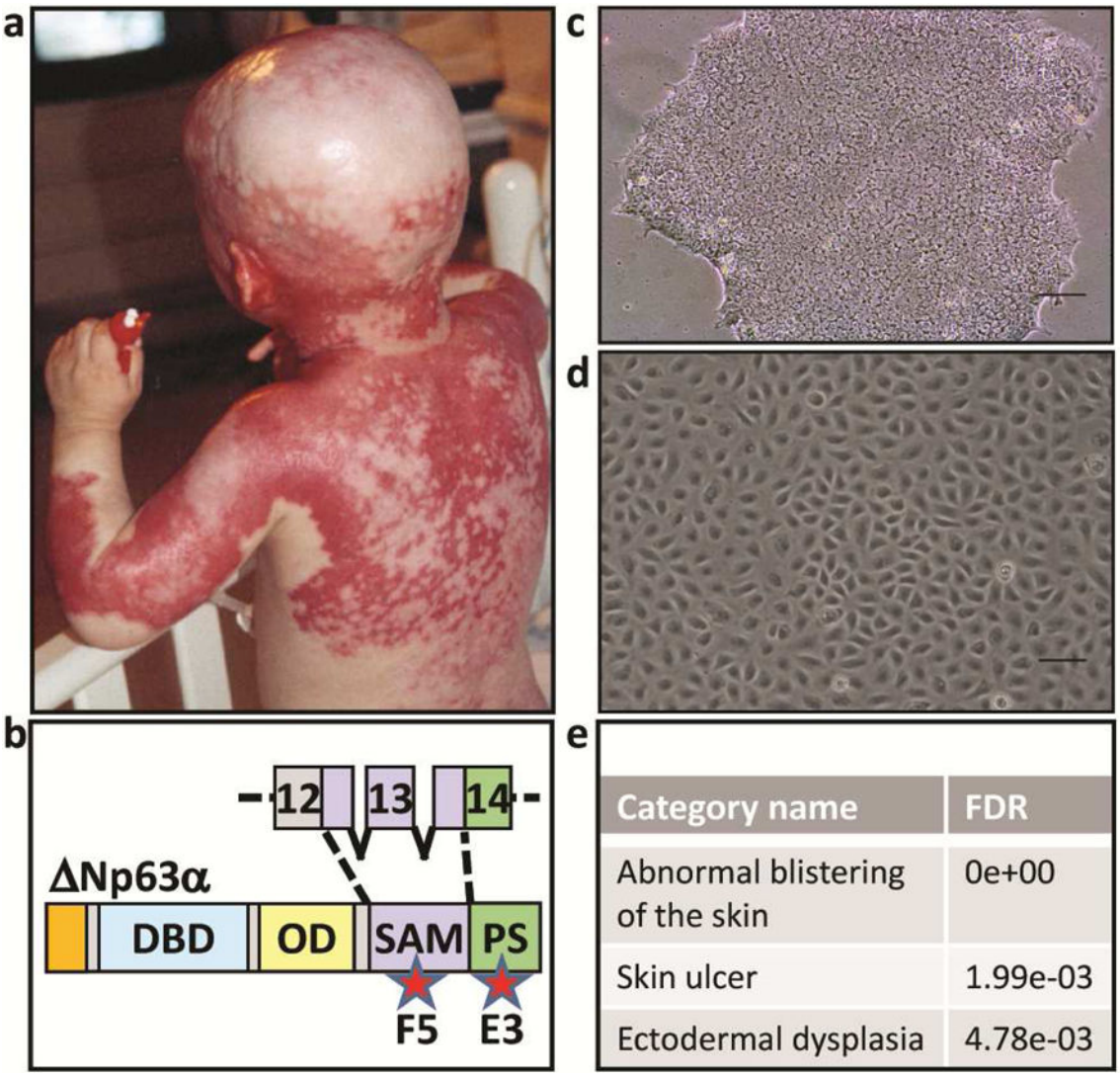

Figure 1. Generating iPSC and iPSC-derived keratinocytes from AEC patient skin

(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 13-14, encoding putative protein-protein interaction domains. Approximate location of mutations in the AEC patient cells (F5 and E3) used in this manuscript are indicated by stars. The protein schematic shown is of ΔNp63α, the predominantly expressed TP63 isoform in human keratinocytes. DBD: DNA binding domain, OD: oligomerization domain, SAM: sterile alpha motif, PS: post-SAM domain. 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-K as determined by a Human Phenotype Ontology analysis of our RNAseq data (FDR; false discovery rate).

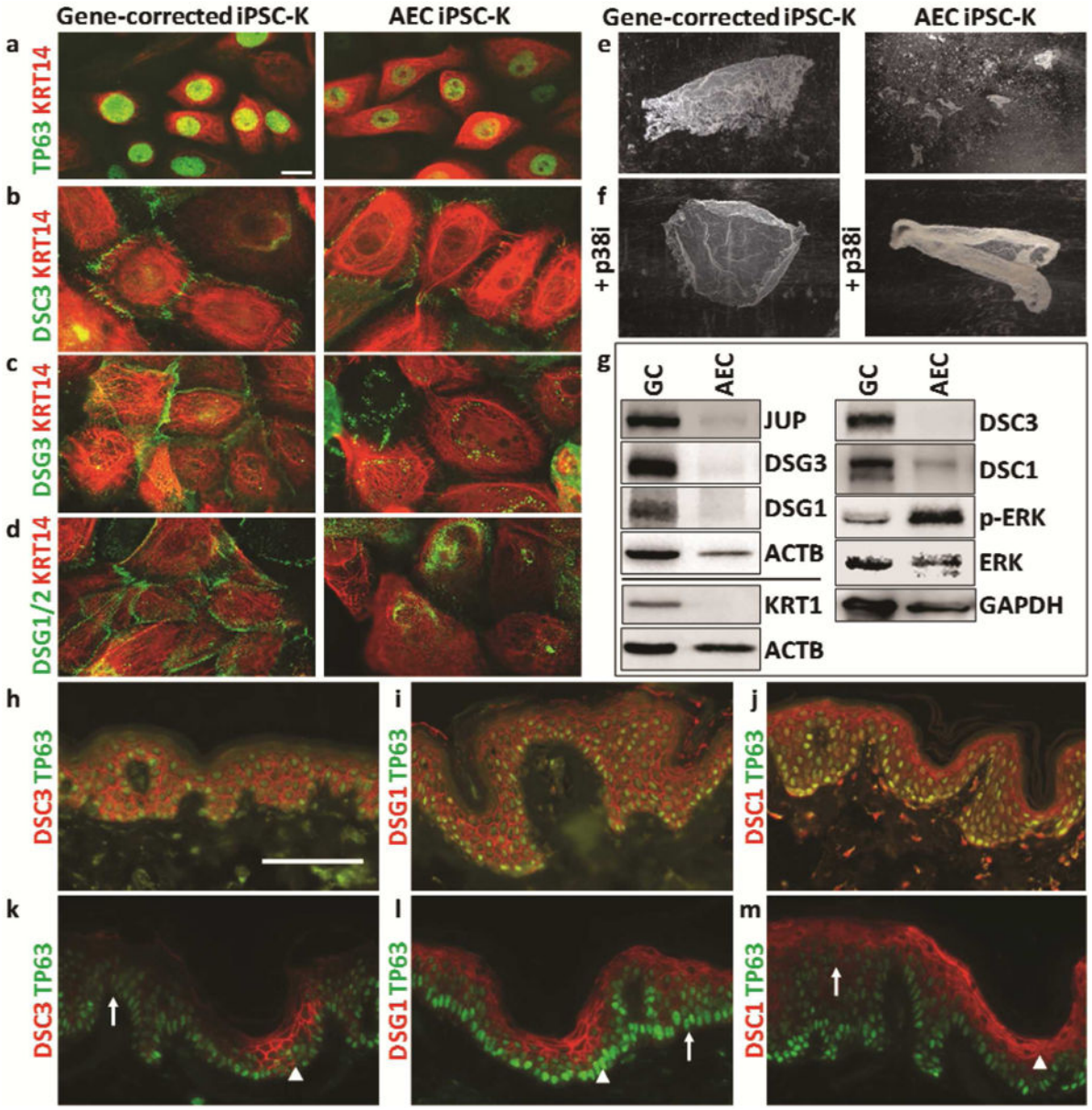

Figure 2. Characterization of desmosomal protein expression in AEC iPSC-K and AEC patient skin

Immunofluorescence analysis of a conisogenic pair of AEC and gene-corrected iPSC-K (F5, F5GC) in low calcium (a) and high calcium media for 24 hours (b-d). (a) TP63 and KRT14, (b) DSC3 and KRT14, (c) DSG3 and KRT14, and (d) DSG1/2 and KRT14 expression and localization. (e) Dispase assay showing accelerated fragmentation of AEC iPSC-K sheets in response to mechanical stress. (f) Treatment with the p38MAPK inhibitor SB202190 prevents AEC iPSC-K sheet fragmentation. (g) Western blot analysis for the indicated proteins of F5 and F5GC iPSC-K after exposure to high calcium conditions for 24 hours. Similar data for E3 and E3GC are shown in Supplemental Figure 2. Note downregulation of several proteins in AEC iPSC-K (JUP, 0.24; DSG3, 0.18; DSG1, 0.41; KRT1, 0.063; DSC3, 0.061; DSC1, 0.49). The ratio of pErk/Erk was increased 4.2 fold in AEC iPSC-K. (h-j)

Immunofluorescence analysis of normal human skin and (k-m) AEC skin for (h,k) DSC3, (i, l) DSG1, and (j, m) DSC1. Arrowheads point towards normal expression while arrows point towards abnormal expression in panels h-j. Size bars: 10μm (a-d), 100μm (h-m)