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Keratinocytes from Induced Pluripotent Stem Cells in Junctional Epidermolysis Bullosa

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Keratinocytes and dermal fibroblasts express adhesive proteins that ensure the epidermis remains attached to the skin basement membrane and to the papillary dermis. Congenital deficiency of any of at least 15 such proteins results in a blistering condition, termed epidermolysis bullosa (EB)(Fine *et al.*, 2008). The most severe form of EB is the Herlitz variant of junctional EB (JEB-H) caused by loss-of-function mutations in one of the three genes (*LAMA3*, *LAMB3*, and *LAMC2*) encoding one of three chains of the heterotrimeric protein laminin 332 (LM-332)(Kiritsi *et al.*, 2011). LM-332 is secreted by keratinocytes and interacts with integrin receptors α3β1 and α6β4 to form focal adhesions and stable anchoring contacts in the dermalepidermal junction (DEJ). Children with this autosomal recessive genodermatosis develop generalized skin blistering; extensive mucosal erosions in the upper respiratory, gastrointestinal, and genitourinary tracts; infections; and, despite supportive measures, typically die within the first year of life.

Even though it has been largely accepted that JEB-H is untreatable(Yuen *et al.*, 2012), evidence from a gene therapy trial for the less severe (non-Herlitz) form of JEB(Mavilio *et al.*, 2006), and from *LAMB3* gene correction of human JEB-H cells –(Robbins *et al.*, 2001; Sakai *et al.*, 2010), suggests novel treatment options.

Prominent among these, the novel technology of reprogramming skin cells into pluripotent stem cells (iPSCs), already applied to EB (Bilousova *et al.*, 2011; Itoh *et al.*, 2011; Tolar *et al.*, 2010) by a combination of specific transcription factors has the dual potential of generating patient-specific, highly proliferative cells for gene-correction strategies and of

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CONFLICT OF INTEREST

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providing a tool for better understanding the biology of JEB-H. We hypothesized that such iPSCs can be derived from JEB-H individuals. Thus, in principle, an inexhaustible supply of patient-specific stem cells can be generated for local wound therapy and for systemic administration aimed at reaching both skin and internal mucosal membranes.

To investigate this, we obtained skin biopsies from two individuals with JEB-H, who carried mutations in the *LAMB3* gene: patient 1 P1; c.1365_1366del (p.Asn456ArgfsX7); c. 2207C>A (p.Ser736X(Varki *et al.*, 2006)) and patient 2 (P2; c.1903 C>T (p.Arg635X); c. 1117C>T (p.Gln373X). Samples were obtained with written informed consent and following a protocol approved by the University of Minnesota Institutional Review Board and with adherence to the Helsinki Guidelines. All mutations create premature stop codons in the open reading frame, with expected nonsense-mediated decay of the mRNA or truncation of the protein product. The children experienced extensive areas of mucocutaneous lesions from birth, hoarseness and stridor, numerous infections, and progressive severe malnutrition. Examination of skin sections revealed the absence of laminin β3 chain at the DEJ. Electron microscopy examination showed infrequent and underdeveloped hemidesmosomes. Collectively these molecular, clinical, biochemical, and ultrastructural features were consistent with the diagnosis of JEB-H.

To derive JEB-iPSCs, we transduced skin fibroblasts with the four transcription factors *OCT4*, *SOX2*, *KLF4*, and c-*MYC*, which are known to induce pluripotency in somatic cells(Tolar *et al.*, 2010). Within three weeks of culture, the patient-specific JEB-iPSCs emerged as raised clusters of cells (Figure 1 a–c). To document the embryonic stem cell-like cellular state, we examined their mRNA and protein expression patterns. When compared with the parental fibroblasts, the JEB-iPSCs expressed the genes coding for nuclear, cytoplasmic, and cell surface proteins (e.g., TRA-1-60, TRA-1-81, stage-specific embryonic antigens 3 and 4, Lin28, Rex1, ABCG2 and DNMT3b, OCT4, and NANOG) in a pattern consistent with embryonic stem cell and iPSC phenotype (Figure 1 d–m). In support of the known activation of endogenous expression of stem cell genes by exogenous reprogramming factors, the maintenance of pluripotency became independent of the original exogenous reprogramming factors (data not shown). As expected in fully reprogrammed iPSCs, the epigenetic profiles showed that endogenous OCT4 and NANOG promoters were largely demethylated (Supplementary Figure 1). The JEB-iPSC lines were maintained for more than 20 passages, and they showed no evidence of genomic instability as evidenced by cytogenetic analysis (Supplementary Figure 2). To exclude the possibilities of cell contamination or the mosaicism observed in JEB(Pasmooij *et al.*, 2007), we verified the authenticity of the JEB-iPSCs by genomic finger-typing with competitive polymerase chain reaction of a variable number of tandem repeat polymorphisms and by sequencing of *LAMB3* gene mutations in the JEB-iPSCs (data not shown). To show that JEB-iPSCs are capable of differentiating into cells of endodermal, mesodermal, and ectodermal origin, we injected them into immune-deficient mice lacking T cells, B cells, and natural killer cells, and having a macrophage defect that makes them reliable recipients of human cells. In 6–8 weeks, cystic teratomas formed and cells derived from all three embryonic layers were seen (Figure 1 n). In aggregate, these data show that fully reprogrammed iPSCs can be derived from skin cells of JEB-H individuals.

JEB-iPSCs can provide means for drug screening and to model cellular interactions among various mucocutaneous cell types derived from the same individual. To our knowledge, this is a previously unreported use of iPSCs as a cellular tool to study the skin pathology in JEB. We showed first that skin-like structures formed in the process of in vivo JEB-iPSC differentiation (Supplementary Figure 3). In contrast to wild-type iPSCs, the skin-like structures arising from the JEB-iPSCs expressed no detectable laminin β3, but expressed collagen type VII, the DEJ protein deficient in distinct, dystrophic forms of EB (Figure 2 a–d). Next, to substantiate the proof-of-concept that skin cell cultures can be derived from JEB-iPSCs, we differentiated JEB-iPSCs into keratinocytes (Supplementary Figures 4 and 5). Lastly, to demonstrate that this operating procedure can serve as a platform for gene correction of these highly proliferative cells, we transduced the JEB-iPSCs with *LAMB3* gene. After transduction, the JEB-iPSC-derived cells expressed and secreted laminin β3 protein (Figure 2 e).

In summary, we have shown that the *LAMB3* defect does not preclude reprogramming into pluripotency, as has been observed in other genetic diseases(Raya *et al.*, 2009). We have also shown that the JEB-iPSCs—in addition to establishing a reliable stem cell source for gene therapy interventions in JEB-H—can be used in the study of early human skin formation and compared to LM-332 in early development. With the ultimate clinical application of iPSC technology in mind, it is worth noting that strategies exist for genome-nonintegrating reprogramming, for depletion of tumor-inducing cells from differentiated iPSC cultures, and—as an JEB-H individual can develop anti-LM-332 antibody—for induction of immunological tolerance to disease-correcting transgenes(Vailly *et al.*, 1998; Wu and Hochedlinger, 2011). Thus, gene-corrected JEB-iPSCs can inform medical advances in this severe and lethal blistering disease, as well as additional extracellular matrix disorders of the skin and other tissues(McGowan and Marinkovich, 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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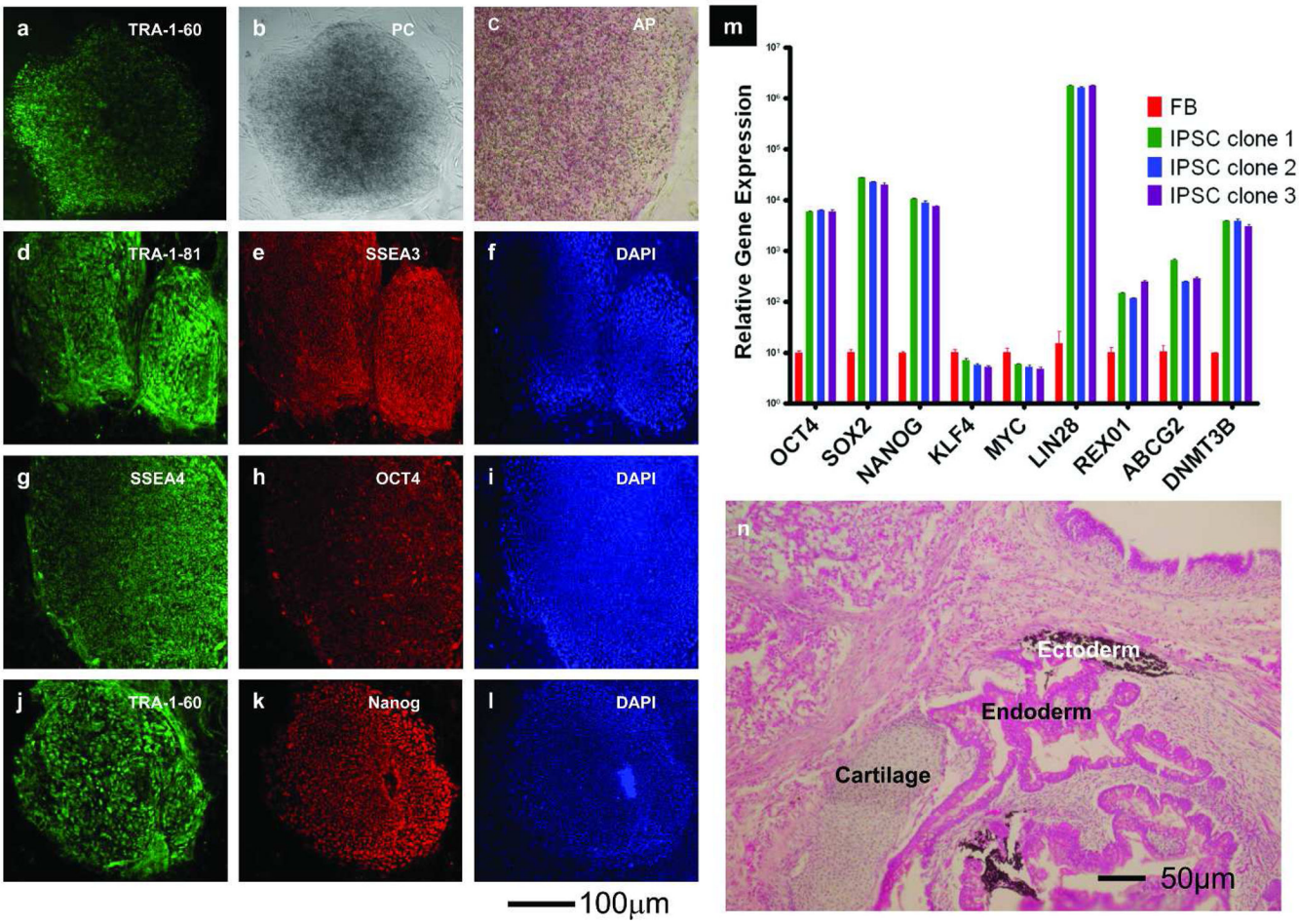

Figure 1. Expression profile of JEB-iPSCs

(**a–l**) To assess the ability of expressing embryonic stem cell proteins, JEB-iPSCs were live stained with TRA-1-60, viewed in phase contrast (PC), immunostained with TRA-1-81, SSEA3, SSEA4, OCT4, TRA-1-60, and NANOG. Corresponding images stained with 4,6-diamidino-2-phenylindole (DAPI) show nuclei of individual cells in the colonies. Scale bar = 100 μm. (**m**) Quantitative reverse transcription PCR analysis of *OCT4*, *SOX2*, *NANOG*, *KLF4*, *c-MYC*, *LIN28*, *REX01*, *ABCG2*, and DNMT3b in parental JEB-H fibroblasts (FB, red bars) and three independent JEB-H iPSC lines (iPSC clones 1, 2, and 3 denoted with green, blue, and violet bars, respectively). All values were normalized against endogenous *GAPDH* expression. (**n**) JEB-H iPSC-derived teratoma differentiated into cells of all three germ lines. Same histologic section of mature teratoma from an immunodeficient mouse shows melanocytes of ectodermal origin, cartilage of mesodermal origin, and columnar epithelium with goblet cells of endodermal origin. Hematoxylin-eosin. The findings were analogous for both P1 and P2, therefore data for P1 are shown here as a representative example. Detailed methods are included as supplementary information.

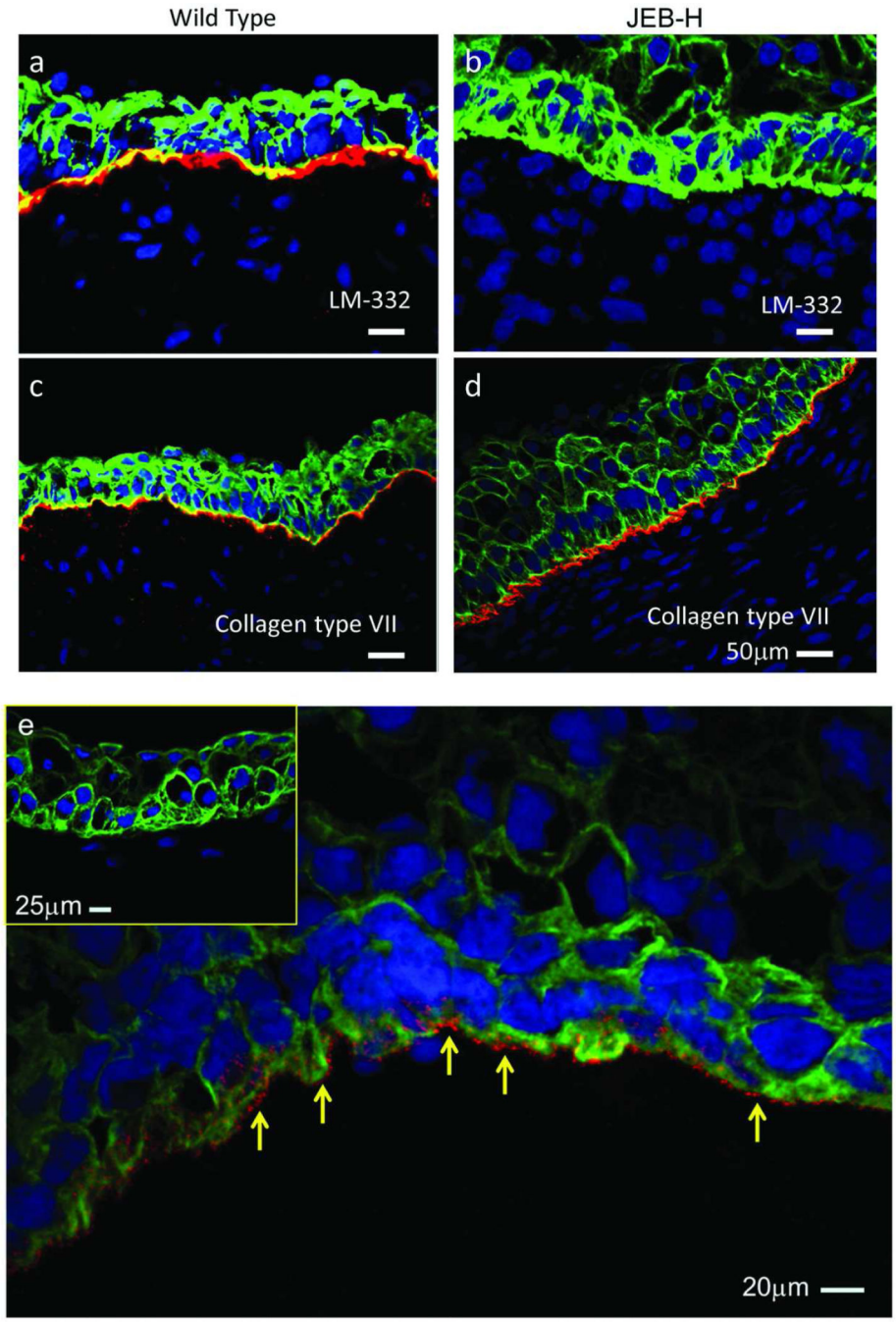

Figure 2. Skin cells derived from JEB-iPSCs

(**a–d**) Immunofluorescent staining of JEB-H iPSC-derived skin-like structures showed that both wild-type and JEB-H iPSC-derived cells stained for collagen type VII (red, bottom panels), but only wild-type skin-like structures were positive for LM-332 (red, top panels). Scale bar = 50 μm (**e**) In contrast to JEB-H skin-like structures (inset), skin-like structures derived in vivo from LAMB3-corrected JEB-H iPSCs expressed extracellular laminin 332 (arrows). Nuclei are stained with DAPI (blue) and keratinocytes are stained with cytokeratin

5 antibody in all the images shown. Detailed methods are included as supplementary information. Scale bar = 20 μm