

Keratinocytes from Induced Pluripotent Stem Cells in Junctional Epidermolysis Bullosa

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TO THE EDITOR

Keratinocytes and dermal fibroblasts express adhesive proteins that ensure the epidermis remains attached to the skin basement membrane and 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 $\alpha3\beta1$ and $\alpha6\beta4$ to form focal adhesions and stable anchoring contacts in the dermal-epidermal 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 technology of reprogramming skin cells into induced pluripotent stem cells (iPSCs), already applied to EB (Tolar *et al.*, 2010; Bilousova *et al.*, 2011; Itoh *et al.*, 2011) by a combination of specific transcription

factors, has the dual potential of generating patient-specific, highly proliferative cells for gene-correction strategies and of 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 performed skin biopsies in 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 messenger RNA 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 $\beta3$ 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 junctional EB-induced pluripotent stem cells (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 3 weeks of culture, the patient-specific JEB-iPSCs emerged as raised clusters of cells (Figure 1a–c). To document the embryonic stem cell-like cellular state, we examined their messenger RNA 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 1d–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 S1 online). 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 S2 online). 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 PCR of a variable number of tandem repeat polymorphisms and by sequencing of *LAMB3* gene mutations in

Abbreviations: DEJ, dermal-epidermal junction; EB, epidermolysis bullosa; iPSC, induced pluripotent stem cell; JEB-H, Herlitz variant of junctional EB; JEB-iPSC, junctional EB-iPSC; LM-332, laminin 332

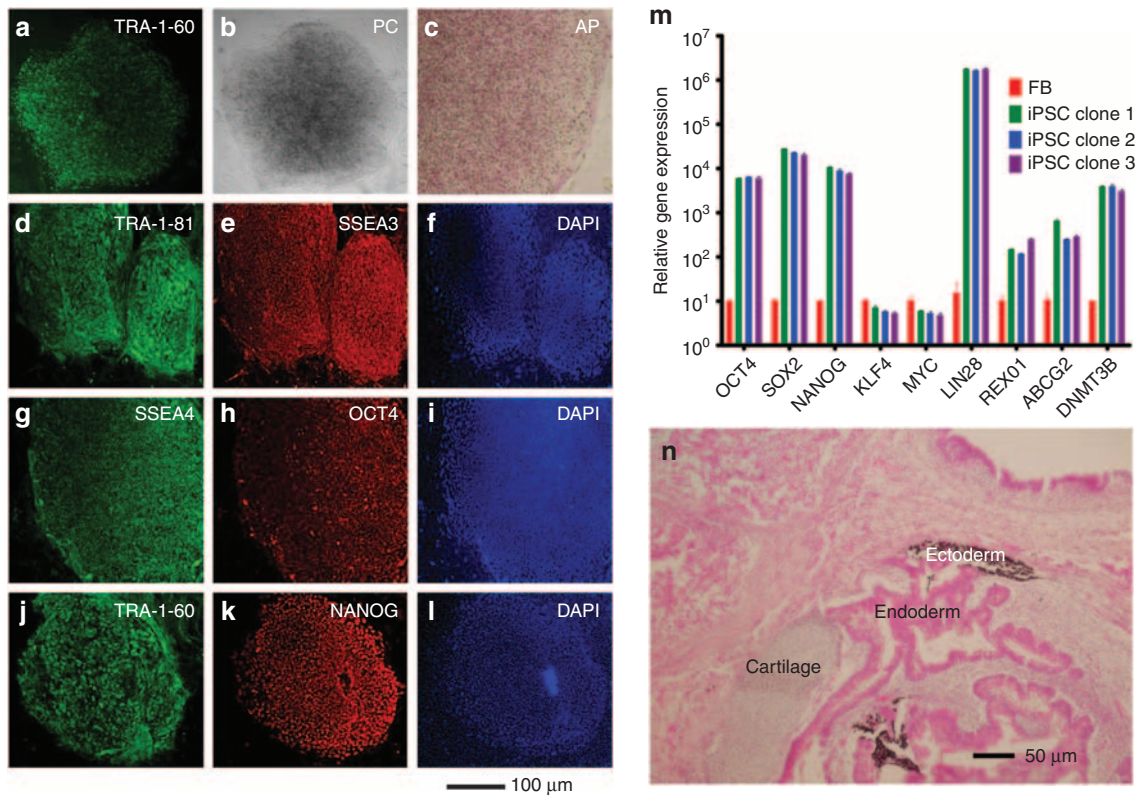


Figure 1. Expression profile of junctional epidermolysis bullosa-induced pluripotent stem cells (JEB-iPSCs). (a–l) To assess the ability of expressing embryonic stem cell proteins, JEB-iPSCs were live stained with TRA-1-60, viewed in phase contrast (PC), and immunostained with TRA-1-81, SSEA3, SSEA4, OCT4, TRA-1-60, and NANOG. Corresponding images stained with 4,6-diamidino-2-phenylindole (DAPI) show the nuclei of individual cells in the colonies. Bar = 100 μ m. (m) Quantitative reverse transcription PCR analysis of *OCT4*, *SOX2*, *NANOG*, *KLF4*, *c-MYC*, *LIN28*, *REX01*, *ABCG2*, and *DNMT3b* in parental Herlitz variant of junctional EB (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 glyceraldehyde-3-phosphate dehydrogenase expression. (n) JEB-H iPSC-derived teratoma differentiated into cells of all three germ lines. The same histological 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 online. AP, alkaline phosphatase.

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 immunodeficient 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 1n). 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 were formed in the process of *in vivo* JEB-iPSC differentiation (Supplementary Figure S3 online). In contrast to wild-type iPSCs, the skin-like structures arising from the JEB-iPSCs expressed no detectable laminin β 3, but expressed collagen type VII, the DEJ protein deficient in distinct, dystrophic forms of EB (Figure 2a–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 S4 and S5 online). 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 LM-332 heterotrimeric protein (Figure 2e).

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 with LM-332 in early development. With the ultimate clinical application of iPSC technology in mind,

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