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Characterization of the epidermal-dermal junction in human induced pluripotent stem cell-derived skin organoids

Veronika Ramovs¹, Hans Janssen², Ignacia Fuentes^{3,4}, Amandine Pitaval⁶, Walid Rachidi⁶,
Susana M Chuva de Sousa Lopes^{1,7}, Christian Freund^{1,5}, Xavier Gidrol⁶, Christine L Mummery^{1,5},
Karine Raymond^{1,5,6*}

¹ Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands.

² Electron microscopy facility, Netherlands Cancer Institute, Amsterdam, The Netherlands

³ Fundación DEBRA Chile, Santiago, Chile.

⁴ Centro de Genética y Genómica, Facultad de Medicina, Clínica Alemana Universidad del Desarrollo, Santiago, Chile.

⁵ LUMC hiPSC Hotel, Leiden University Medical Center, Leiden, The Netherlands.

⁶ University of Grenoble Alpes, CEA, INSERM, IRIG-BIOMICS, Grenoble, France.

⁷ Ghent Fertility and Stem cell Team (G-FaST), Department for Reproductive Medicine, Ghent University Hospital, Ghent, Belgium.

* Corresponding author. E-mail address: k.i.raymond@lumc.nl

SUMMARY

Human induced pluripotent stem cell (hiPSC)-derived hair-bearing skin organoids offer exciting new possibilities for modelling diseases like epidermolysis bullosa (EB). These inherited diseases affect 1 in 30,000 people worldwide and result from perturbed expression and/or structure of components of the epidermal-dermal junction (EDJ). To establish whether hiPSC-derived skin organoids might be able to capture salient features of EB, it is thus important to characterize their EDJ. Here we report successful generation of hair-bearing skin organoids from two hiPSC lines that exhibited fully stratified interfollicular epidermis. Using immunofluorescence and electron microscopy, we showed that basal keratinocytes in organoids adhere to laminin-332 and type IV collagen-rich basement membrane via type I hemidesmosomes and integrin β 1-based adhesion complexes. Importantly, we demonstrated that EDJs in organoids are almost devoid of type VII collagen, a fibril that mediates anchorage of the epidermis to dermis. This should be considered when using skin organoids for EB modelling.

INTRODUCTION

Skin integrity depends on the epidermal-dermal junction (EDJ), the interface between basal keratinocytes of the epidermis and stroma of the dermis. Basal keratinocytes adhere to the underlying basement membrane (BM), an extracellular matrix (ECM) sheet, composed primarily of laminin and type IV collagen (ColIV) networks (Burgeson and Christiano, 1997). This adhesion is mediated by integrins, transmembrane $\alpha\beta$ heterodimer proteins that cluster to adhesion structures on the basal plasma membrane of keratinocytes. In physiological conditions, keratinocytes highly express collagen-binding integrin $\alpha2\beta1$, laminin-332/-511-binding $\alpha3\beta1$ and laminin-332-binding $\alpha6\beta4$. The adhesive integrity of EDJ largely depends on $\alpha6\beta4$ -containing adhesion structures called hemidesmosomes (HDs). In contrast to simple epithelia (e.g. intestine), which express type II HDs consisting solely of the integrin $\alpha6\beta4$ and plectin, keratinocytes in EDJ adhere via type I HDs that additionally contain tetraspanin CD151 and bullous pemphigoid antigens 180 (BP180) and 230 (BP230) (Margadant et al., 2010). Additionally, BM is connected to the dermal connective tissue via anchoring fibrils, consisting of type VII collagen (ColVII) (Burgeson and Christiano, 1997).

Aside from its function in epidermal adhesion, EDJ also regulates the permeability barrier of the skin and plays a role in epithelial-mesenchymal interactions and signal transduction. Its pivotal role in skin physiology is reflected in severe pathologies that occur when the function of EDJ components is impaired. This is the case in for example epidermolysis bullosa (EB), which is caused by, among others, mutations in genes encoding components of HDs and associated filaments. These include mutations in the *COL7A1* gene (coding for ColVII) causing dystrophic EB, a severe blistering disease (Turcan and Jonkman, 2015). Furthermore, EDJ, particularly BM and integrins, play an important role in initiation and progression of skin cancers (Chang and Chaudhuri, 2019; Ramovs et al., 2016).

The generation of hair-bearing skin organoids derived from human pluripotent stem cells (hPSC) has recently been described (Lee et al., 2020). These clearly offer exciting new opportunities for skin disease modeling, regenerative medicine and developmental research. However, to grasp the full potential of this model, it is essential to understand the structure and developmental stage of EDJs formed in the skin organoids. This is especially important since their gene expression profile and the hair morphology indicate that they represent mid-gestational skin, a fetal stage during which the main components of EDJ gradually begin to develop (Nicholas A. and Jacques P., 2005). In addition, inherent differences between hPSC lines can lead to variable outcomes (Lee and Koehler, 2021) and therefore require the validation of protocols with independent cell lines.

In this study, we report the generation of hair-bearing skin organoids from two male human induced pluripotent stem cell (hiPSC) lines and address the organization of their EDJ using immunofluorescence (IF) and transmission electron microscopy (TEM).

RESULTS & DISCUSSION

Successful generation of hair-bearing skin organoids from two hiPSC lines

Using two independent control hiPSC lines (LUMCi045-A1 and LUMCi046-A1), we were able to recapitulate the *in vitro* skin organogenesis described by Lee et al. (Lee et al., 2020) (Fig. 1a). Consistent with the original protocol, the majority of skin organoids became bipolar at around day 30 of directed differentiation, with an opaque cell mass ('tail') at the pole opposite to the translucent epidermal cyst ('head') (Fig. 1a). At around day 50, the epithelium was composed of a basal layer of keratin 5 (KRT5)⁺ keratin 15 (KRT15)⁺ cells, an intermediate KRT5^{low} layer and a KRT5^{low} KRT15^{high} periderm-like layer (Fig. 1b). Hair placodes were observed at around day 60 and fully grown HFs developed after 100 days in culture (Fig. 1a-

c). At day 130, 78.9% and 75.7% of the skin organoids derived from LUMCi045-A1 and from LUMCi046-A1 displayed HFs, with a mean number of 18 and 16 HFs per organoid, respectively (Fig. 1d). HFs showed similar organization to that previously reported in organoid-derived HFs, occasionally containing sebum-producing sebaceous glands (Fig. 1c, Fig. S1a-b) and innervation as illustrated by TUJ1⁺ neuronal processes and TUJ1⁺ Merkel-like cells (Fig. S1c) (Lee et al., 2020). When pigmented, the matrix region of the HFs and interfollicular epidermis were enriched with PMEL⁺ melanocytes (Fig. S1d-e). Skin organoids were rich in vimentin⁺ dermal fibroblasts, which, much like mid-gestational fetal skin, clustered to FAP^{high} papillary fibroblasts in the upper dermis and CD90^{high} FAP^{low} reticular fibroblasts in the lower dermis (Fig. 1e, Fig. S1d) (Korosec et al., 2019). Consistent with the previous report, we also observed hyaline cartilage in all organoids analysed (Fig. S1f) (Lee et al., 2020).

hiPSC-derived skin organoids form a stratified interfollicular epidermis

At day 130, skin organoids formed a stratified epidermis, which resembles the stratification of the adult human skin: basal keratinocytes express KRT5 and KRT14 and suprabasal keratinocyte layers contain KRT1 and KRT10, with a terminally differentiated loricrin-positive cornified layer marking the upper end of the epidermis (Fig. 1c, f). Such complete stratification of organoid interfollicular epidermis was shown previously when mature organoids were grafted in a mouse model (Lee et al., 2020) but our data support the notion that organoid grafting may not be necessary for short-term experiments that require mature stratification. Nonetheless, it is important to note that organoid grafting in mice leads to the formation of rete-ridge-like structures, which are present in human skin but which neither we nor Lee et al (Lee et al., 2020) observed in the epidermis of skin organoids (Fig. 1f). As rete ridges develop after week 19 of gestation (Fig. 1f), this likely indicates increased maturity of the model after

grafting. Furthermore, mechanical stress has been shown to play an important role in the development of rete ridges (Penrose and Ohara, 1973; Xiong et al., 2013), thus their absence might also reflect the lack of mechanical stress in the *in vitro* culture.

Basement membrane underlies the epidermis of hiPSC-derived skin organoids

Basal keratinocytes adhere to the BM, an ECM sheet composed of CollIV and laminin networks, which can be detected as early as week 7 of fetal development (Hertle et al., 1991). Epidermal BM predominantly contains laminins-332 and -511, of which laminin-332 functions as a main anchoring filament for basal keratinocytes (Burgeson and Christiano, 1997; Pozzi et al., 2017). In organoids, both laminin-332 and CollIV showed homogeneous expression, restricted to the BM (Fig. 1f, 2a). Consistent with their role in adhesion to the BM, all subunits of the major epidermal integrins could be detected at the EDJ in the epidermal cyst of organoids: $\alpha 3$, $\alpha 6$, $\beta 4$ and $\beta 1$ (Fig. 2b-c). In adult skin, integrin $\alpha 6\beta 4$ mainly localizes to the basal membrane of basal keratinocytes, whereas its expression is pancellular early during fetal development, with progressive restriction to the basal membrane from week 10 of gestation (Hertle et al., 1991). The stratified epidermis of immature organoids (day 55) resembles early stages of skin development with strong expression of integrin $\alpha 6\beta 4$ also at the lateral and apical membranes of basal keratinocytes (Fig. S2); this becomes more restricted to the basal membrane in mature organoids (day 130), reflecting the organization of human skin from mid-gestation onwards (Fig. 2b). Integrin $\alpha 3\beta 1$ can be observed at basal and lateral membrane of keratinocytes at EDJ of organoids, which is in line with its localization in mature epidermis (Fig. 2b, white arrows) (Zuidema et al., 2020).

Next, we examined the expression of ColVII, which anchors BM to the dermis, using polyclonal and monoclonal antibodies. While homogenous and abundant in adult skin, ColVII expression

was rather heterogenous and barely detectable along the EDJ of skin organoids. By contrast, all other ECM components of the EDJ that were examined were clearly detectable, albeit at reduced levels compared to human skin (Fig3a-b, Fig. S3a). In line with this, the expression of *COL7A1* was lower in organoids compared to adult human skin when normalized to the expression of two chains encoding laminin-332 and two chains encoding *COL4V* (Fig. S3b).

The low levels of ColVII are remarkable, considering that weak expression of this protein has been detected as early as at 8-weeks of gestation in fetal skin and homogeneous interfollicular expression has been observed from 13 weeks of gestation onwards (Karelina et al., 2000; Ryyänen et al., 1992). Indeed, analysis of 19-week-old fetal skin revealed comparable levels of ColVII to those in adult human skin (Fig. 3a-b). Given that skin organoids resemble fetal skin of gestational week 18 (Lee et al., 2020), low and heterogeneous levels of expression of ColVII were not expected. To rule out any specific defects in the hiPSC lines used in this study, we quantified the expression of ECM components in organoids generated from previously published female hiPSC line WT2. The levels of ColVII in WT2 organoids were similar to those observed in LUMCi045-A1 and LUMCi046-A1 (Fig. S3c-d). We further differentiated LUMCi045-A1 and LUMCi046-A1 directly to keratinocytes and compared their levels of ColVII with those in primary human keratinocytes. When grown on glass coverslips, hiPSC-derived and primary keratinocytes produced comparable amounts of ColVII (Fig. 3c-d, Fig. S3e-i), suggesting that low levels of ColVII observed in organoids are model-dependent. Remarkably, skin organoids did not show obvious morphological defects in the EDJ area, which would be expected in the light of weak anchoring of the epidermis. This could be explained by the lack of exposure to mechanical stress; furthermore, low mechanical stress could by itself affect the assembly of collagen fibrils (Kubow et al., 2015). It would be interesting to determine whether

aberrant expression of ColVII could be resolved by the maturation and/or changes in mechanical stress; analysis of grafted organoids could be informative in this regard. Importantly, the aberrant levels of ColVII could be a limiting factor for the use of skin organoids for *in vitro* disease modeling: its low expression does not only result in severe types of EB but ColVII also plays an important role in wound healing and epidermal squamous cell carcinoma (Chung and Uitto, 2010; Nyström et al., 2013).

Basal keratinocytes in skin organoids adhere to the basement membrane via type I hemidesmosomes and integrin β 1-based adhesion complexes

Firm adhesion of keratinocytes to the BM is mediated by HDs, which link the keratin cytoskeleton to the laminin-332 via the integrin α 6 β 4. Since impaired function of virtually any HD component leads to the development of EB, we thought it was imperative to investigate to what extent skin organoids form HDs (Margadant et al., 2010). Using TEM, we detected numerous HDs localized at the basal membrane of the keratinocytes in EDJ (Fig. 4a). Next, we investigated the expression of BP230, a plakin that in addition to plectin connects integrin α 6 β 4 to keratin cytoskeleton in more complex type I HDs, which are normally found in the epidermis (Margadant et al., 2010). We observed colocalization of BP230 with HD components α 6 β 4, plectin and laminin-332, confirming the presence of type I HDs in organoids (Fig. 4b).

Integrins consisting of β 1 subunit, particularly α 3 β 1 and α 2 β 1 also contribute to the adhesion of basal keratinocytes by linking the ECM to the actin cytoskeleton via adaptor proteins talin and vinculin (Margadant et al., 2010). Whereas vinculin is restricted to the BM in adult skin, its expression can also be observed at the lateral and apical membranes of basal and suprabasal keratinocytes in skin organoids (Fig. 4c); this resembles vinculin localization in skin at mid-gestation of human fetal development (Fig. S4) (Hentula et al., 2001). Integrin α 3 β 1-

and $\alpha 2\beta 1$ -containing adhesion structures also play a role in migration and spreading of keratinocytes during non-homeostatic conditions, such as wound healing, as well as in maintaining cell-cell contact integrity (Zuidema et al., 2020). Keratinocytes in epidermis form cell-cell adhesions via E-cadherin (E-cad)⁺ adherens junctions, Desmoglein-3 (dsg-3)⁺ desmosomes, Zonula Occludens-1 (ZO-1)⁺ tight junctions and Connexin 43 (Cx43)⁺ gap junctions (Churko and Laird, 2013; Sumigray and Lechler, 2015). All main adhesion junctions were observed in skin organoids at expected sites (Fig. 3a, d-f), further confirming the relatively mature state of skin stratification.

In summary, we were able to generate hair-bearing skin organoids from two independent hiPSC lines following the multistep protocol recently developed by Lee et al. (Lee et al., 2020). Moreover, we thoroughly characterized their EDJ. We showed that skin organoids form a fully stratified interfollicular epidermis *in vitro*. Basal keratinocytes in organoids adhere to laminin-332 and ColIV-rich BM via type I HDs and integrin $\beta 1$ -based adhesion complexes. EDJ in organoids derived from our cell lines was almost devoid of ColVII, indicating that further maturation is required to take full advantage of skin organoids as disease model for some forms of EBs, in particular those caused by mutations in the *COL7A1* gene (Table S1).

EXPERIMENTAL PROCEDURES

Cell culture

LUMCi045-A1, LUMCi046-A1 and WT2 hiPSC lines were previously described (Ramovs et al., 2021; Zhang et al., 2014). They were maintained in StemFlex™ medium (ThermoFisher Scientific, #A3349401) on vitronectin-coated plates (Stemcell Technologies, #07180) and passaged twice a week as single cells by using Gentle Cell Dissociation Reagent (Stemcell Technologies, #07174) at the density of 2.5×10^4 cells/cm². Keratinocytes were differentiated

from LUMCi045-A1 and LUMCi046-A1 as described previously and cultured in CnT-07 (Bio-Connect) (Guo et al., 2013, supplemental experimental procedures). Primary human keratinocytes were isolated from breast skin tissue explants as previously described (Auxenfans et al., 2012) and cultured in serum-free keratinocyte medium (ThermoFisher Scientific, #17005042) supplemented with 50µg/ml bovine pituitary gland extract, 2.5ng/ml EGF and antibiotics. Donors signed informed consents and the study was approved by the French research ministry (DC-2020-4338). HaCaT keratinocytes were cultured in CnT-07. All cell lines were kept at 37 °C, 5% CO₂ and 20% O₂.

Generation of skin organoids and quantification of HF formation frequencies

Hair-bearing skin organoids were generated as previously described (Lee and Koehler, 2020). Skin organoids that did not show bipolar organization at around day 30 were excluded from the study. Quantifications of the percentage of organoids producing HFs and of the number of HFs per skin organoid were performed at Day 130 in 3 independent differentiations for both hiPSC lines, respectively. Images of each organoid were taken with a Leica M420 microscope using Olympus XC50 Digital color camera. The angle of the organoids revealing the highest number of HFs was selected for quantification.

Human tissue specimens

Adult human skin samples used in this study were obtained upon mammoplasty or abdominoplasty of females of 32- to 40-year-old with type I/II skin, based on the Fitzpatrick skin type classification scale. The human fetal tissue used in this work (19-weeks, facial area) was obtained from elective abortion (without medical indication) with signed informed consent from all donors. The work described here was reviewed and approved by the Medical Ethical Committee of Leiden University Medical Centre (P08.087).

Antibodies

Primary antibodies used are listed in Table S2 and secondary antibodies are listed in Supplemental Experimental Procedures.

Immunofluorescence microscopy

For cryosections, organoids were embedded in 15% sucrose/7.5% gelatin solution as previously described (Bajanca et al., 2004). Sections (10 μ m thick) were fixed 10min in ice-cold acetone (or 15min in 4% paraformaldehyde when stained with Nile Red) and blocked with 2% bovine serum albumin (BSA, Sigma) in PBS for 1h at room temperature (RT). hiPSC-derived and primary keratinocytes were seeded on coverslips and fixed after 4 days with 2% paraformaldehyde for 30min, permeabilized with 0.2% Triton-X-100 for 5min and blocked with PBS 2% BSA for 1h at RT. Incubations with primary and secondary antibodies were performed in PBS 2% BSA for 1h at RT. For Nile Red staining, sections were further incubated for 15min in Nile Red/PBS (0.05 μ g/mL). Nuclei were stained with DAPI. When indicated, filamentous actin was visualized using Alexa Fluor 647-conjugated phalloidin (ThermoFisher #A22287). Sections were mounted in Fluoromount-G[®], SouthernBiotech and coverslips in Mowiol. For whole-mount immunostaining, organoids were processed as previously described (Lee et al., 2020). Cryosections (human skin and Day 130 organoids) and keratinocytes were analyzed with Zeiss LSM900 Airyscan2 upright confocal microscope and cryosections of Day 55 organoids as well as whole-mounts, with Leica TCS SP5 confocal microscope. Images of maximum intensity projection are presented. Images of skin organoids developed from LUMCi045-A1 hiPSC line are shown. All images were processed using Fiji/ImageJ (Rueden et al., 2017; Schindelin et al., 2012). Detailed explanation of the quantification of the ECM components can be found in Supplemental Experimental Procedures.

Histology

Cryosections of skin organoids were stained for hematoxylin and eosin (H&E). Images were taken with Olympus AX70 microscope using Olympus XC50 Digital color camera.

Transmission electron microscopy

For transmission electron microscopy, tissue samples were fixed in Karnovsky's fixative. Post-fixation was done with 1% Osmiumtetroxide in 0.1 M cacodylatebuffer. Organoids were washed and stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. The samples were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, U.S.A), sectioned and analyzed with a Tecnai12G2 electron microscope (Thermo Fischer, Eindhoven, the Netherlands). Images of skin organoids developed from LUMCi045-A1 hiPSC line are shown.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 9.1.1). Unpaired two-tailed t test was used to compare two experimental groups. Experiments with more than two experimental groups were analyzed using one-way ANOVA. Comparisons were conducted using Tukey's multiple comparison test after a global ANOVA was determined to be significant. Results with P-value lower than 0.05 were considered significantly different from the null hypothesis.

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AUTHOR CONTRIBUTIONS

KR and VR designed and performed the experiments and wrote the manuscript. HJ performed TEM, IF provided samples for the generation of hiPSC lines, AP and WR provided primary keratinocytes and SMCdSL collected and isolated the fetal human skin. KR, CF, XG and CLM provided supervision and discussion. All authors read and approved the manuscript.

DECLARATION OF INTEREST

CLM is associate editor of Stem Cell Reports.

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FIGURE TITLES AND LEGENDS

Figure 1: HFs, dermal fibroblasts and stratification of interfollicular epidermis in hiPSC-derived skin organoids. (a) Representative bright field images of the organoids at different stages of development. Scale bars: 200 μ m (day 0-12) and 400 μ m (day 30-130). (b) Representative bright field (left) and confocal (right) images of organoid sections at day 55, stained with H&E (left) or with anti-KTRT5 and KRT15 antibodies (right). Scale bar: 20 μ m. (c) Representative bright field image of H&E-stained section of skin organoid at day 130 showing stratified epidermis and fully developed HF, equipped with sebaceous gland (arrow). (d) Quantification of the percentage of organoids with HFs (left) and the number of HFs per organoid (right) derived from LUMCi045-A1 (045-A1) and LUMCi046-A1 (046-A1) lines at day 130. The mean \pm SD of 48-70 organoids from 3 independent differentiations is shown. (e) Characterization of dermal fibroblasts in organoid (day 130) and fetal human skin sections.

Vimentin⁺ fibroblasts cluster with the FAP⁺ population in the upper dermis and FAP^{low} CD90⁺ population in the lower dermis (delineated with dashed line). Upper dashed line: BM. Scale bar: 100 μ m. (f) Confocal images of organoid (day 130) and adult and fetal human skin sections stained with anti-laminin-332 antibody as well as with anti-KTRT14, KRT5, KRT10, KRT1 or loricrin antibodies. Note that the stratification of the skin organoids (top) is comparable to that of human epidermis (bottom). Arrow: rete ridges, present only in adult human skin. Scale bars: 20 μ m.

Figure 2: Characterization of the basement membrane of hiPSC-derived skin organoids. (a) Representative confocal images of organoid and human adult and fetal skin sections stained with anti-Collagen IV and anti-laminin-332 antibodies. Scale bars: 40 μ m. (b) Representative confocal images of organoid and human skin sections stained for laminin-332 in combination with keratin 5 (KRT5) and integrin β 4 subunit (left panels), integrin β 1 and integrin β 4 subunits (middle panels) or integrin α 6 and integrin α 3 subunits (right panels). The distribution of laminin-332 and integrin subunits α 3, α 6, β 4 and β 1 in skin organoids is similar to that in adult and mid-gestational fetal human skin. Arrows point to the localization of integrin α 3 β 1 to cell-cell contacts. Scale bars: 40 μ m. (c) Confocal images of organoid whole mounts stained with anti-KRT5 and anti- β 4 integrin antibodies (left panels) or with anti-vimentin and anti- α 3 and - α 6 integrin subunits antibodies (right panels). Note the homogeneous distribution of integrins α 3 β 1 and α 6 β 4 to the polarized head of organoids. Scale bars: 200 μ m (a-c) Organoids are imaged at day 130. Dashed boxes are magnified. Confocal images are presented as maximum projected z-stacks.

Figure 3: Expression levels of colVII in skin organoids

(a) Representative confocal images of organoid (day 130) and human fetal and adult skin sections stained with anti-laminin-332 and anti-ColVII antibodies. The expression of colVII is low in the skin organoids and often non-uniform (arrows), with some areas seemingly devoid of any colVII fibers (asterisk). The panels 'background control' represent the signal observed when using secondary antibodies only, with similar setting to those applied for the condition 'increased laser power'. Dashed line marks the BM. Dashed boxes are magnified. Confocal images are presented as maximum projected z-stacks. (b) Quantification of the laminin-332, colIV and colVII mean intensity (MI) in skin organoids (day 130) derived from LUMCi045-A1 (left), LUMCi046-A1 (middle) lines and fetal skin (right) as compared to adult human skin. A total of 5-6 images of 2-4 independent differentiations of hiPSC or of human samples was used for quantifications (Tukey's multiple comparison, $n = 12-23$, $**P < 0.01$, $****P < 0.0001$, mean \pm SD). Scale bar: 100 μ m. (c) Representative images of primary and hiPSC-derived keratinocytes stained with anti-colVII and integrin β 4 antibodies and phalloidin. Scale bars: 30 μ m. (d) Violin plots showing the colVII expression of hiPSC-derived keratinocytes as a percentage of colVII mean intensity (MI) of primary keratinocytes. Line: median, dashed lines: quartiles, $n=142-223$ β 4-positive cells.

Figure 4: Basal keratinocytes adhere to the basement membrane via type I hemidesmosomes. (a) Representative TEM images of the organoid and adult human epidermis. HDs (asterisk) are observed at the basal membrane of the basal keratinocytes (blue), adhering to the underlying basement membrane (dashed line). Desmosomes can be detected in cell-cell adhesions sites in different layers of the stratified epidermis. (b) Representative confocal images of skin organoid showing colocalization of plectin, β 4 integrin, BP230 and Laminin-332. This identifies HDs in organoids as type I, which are typically found in

the adult human skin. **(c)** Representative confocal images of organoid and human skin sections stained with anti- $\alpha 6$ integrin, anti-Laminin-332 and anti-vinculin antibodies. Whereas vinculin is restricted to the BM in the adult skin, its expression can be observed also at the lateral and apical membranes of basal and suprabasal keratinocytes in skin organoids (arrowhead), which is similar to that observed during the mid-gestation period of human fetal development (Fig. S4). **(d-f)** Representative confocal images of skin organoids stained for desmosomal marker-desmoglein-3 (dsg-3) and integrin $\alpha 6$ together with **(d)** gap junction marker connexin 43 (Cx43), **(e)** adherens junction marker E-cadherin (E-cad) or **(f)** tight junction marker ZO-1. Note the lower expression of dsg-3 in upper layers of epidermis. Dsg-3, Cx43, E-cad and ZO-1 localize to the cell-cell junctions. Dashed boxes are magnified to the right. **(b-e)** Scale bars: 40 μ m. Confocal images are presented as maximum projected z-stacks. All organoids are imaged at day 130.







