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Mechanical stretching stimulates growth of the basal layer and rete ridges in the epidermis

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A procedure called tissue expansion (TE) is used in reconstructive surgery to amend congenital or trauma-induced skin defects. During TE, a silicone expander is placed under the skin and gradually filled with saline to exert mechanical stretching (Zollner, Buganza Tepole, Gosain, & Kuhl, 2012; Zollner, Holland, Honda, Gosain, & Kuhl, 2013). Typically, TE takes about three months, and saline is added weekly. When moderately applied, TE stimulates skin growth, resulting in extra tissue that matches local characteristics. Although the procedure is commonly used in surgery, complications such as inflammation, necrosis, or scarring occur in up to 40% of the time (Bjornson, Bucevska, & Verchere, 2019; Karimi et al., 2019; LoGiudice & Gosain, 2003). TE is particularly difficult in anatomical regions such as the scalp and extremities (LoGiudice & Gosain, 2003; Tepole, Gosain, & Kuhl, 2012) suggesting that unknown factors limit mechanical stimulation, and necessitating more study. Porcine skin is similar to human skin, and these similarities are particularly evident in the epidermis, as both include undulated epidermal projections called rete ridges (RRs) (Penrose & Ohara, 1973; Rittie, 2016). The significance of RR in epidermal maintenance is not well recognized, in humans, the appearance of RR changes with age and health conditions; RR are diminished in height in aging skin (Giangreco, Goldie, Failla, Saintigny, & Watt, 2010; Langton, Graham, Griffiths, & Watson, 2019), and are enlarged by diseases like psoriasis (Fraki, Briggaman, & Lazarus, 1983). Therefore, we employed a minipig model to study the effects of TE on epidermal structure. Our results indicate that acute skin stretching introduces morphogenetic changes in the epidermis. Mechanical force triggers a rapid proliferation of basal precursors leading to growth of the basal layer and enlargement of RR. This mechanism protects the layered organization of the epidermis and increases the regenerative potential of expanded skin. Thus, brief mechanical stimulation could be

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CONFLICT OF INTEREST

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considered as part of therapeutic procedures when an expansion of epidermal progenitors is needed.

We have previously developed a TE model using minipig (Purnell et al., 2018). All TE procedures were performed on a Yucatan strain of minipig. Three 5–6 week old females were fitted with two identical expanders placed subcutaneously on the back skin, one over the ribs, and the second over the abdomen. Expanders were placed under general anesthesia, the post-operative pain and distress were monitored and treated in accordance with IACUC protocol LCH14–006. After one week of recovery from the surgery, the expanders were inflated using remotely located external ports (LoGiudice & Gosain, 2003), each with 60 ml saline at 1 hour, 24 hours, 3 days, and 7 days before the animals' sacrifice. In addition, a repeated stretch (TE-7+7 days), was developed by injecting the same volume of saline a second time, extending TE to 14 days. Punch biopsies from the apex of the expanded skin, and controls collected from the contralateral sites were preserved in paraffin, tissue sections (4 μ m) were used for histology and immunostaining (Petridou & Skourides, 2016).

Maintenance of a healthy epidermis depends on a population of precursors that adhere to an underlying basement membrane (BM) through integrin receptors (Brakebusch et al., 2000). Moreover, mechanical force can activate β 1 integrin at the lateral region of the cell cortex and change the orientation of the mitotic spindle and cell division (Petridou & Skourides, 2016). Accordingly, we hypothesized that during TE, β 1 integrin will relocate dynamically, in order to adapt the basal cell to mechanical stimulation. If these changes promote symmetrical cell division than the basal layer would elongate, as evaluated on histological sections.

Consistent with the literature (Brakebusch et al., 2000; Hotchin, Gandarillas, & Watt, 1995; Jones, Harper, & Watt, 1995; Tan et al., 2013), we found that the normal porcine interfollicular epidermis (IFE) accumulates β 1 integrin heterogeneously (Fig. 1 a). Cells at the tips of the RRs accumulated a relatively low level of the protein, but some basal cells covering the tips of the dermal papillae were strongly positive, suggesting that they may have been stem cells. However, after a one-hour stretch (TE-1h) larger clusters of negative cells were detected at RR tips (Fig. 1 b, green arrows), implying that the initial stretch caused detachment of some cells from the BM (Hotchin et al., 1995). In cells covering the tips of the dermal papillae, β 1 integrin occurred in stripes between adjacent precursors (Fig. 1 b, black arrows) indicative of basolateral cortex accumulation. Furthermore, at TE-1h, we found β 1 integrin at the apical site of mitotic precursors, with a frequency almost four times higher than in the control ($p=0.0001$) (Fig. 1 b, red arrows) suggesting reversed cell polarity (Clayton et al., 2007; Lechler & Fuchs, 2005). This effect gradually normalized, showing a $50\pm 15\%$ reduction by 24 hours (TE-24h, $p=0.003$). By 3 days of TE, β 1 integrin almost disappeared from the apical localization (Fig. 1 c - d) and, became visible along the entire basal layer in a diffused manner (TE-7d and TE-7+7d). Stripes between cells were now rarely detected (Fig. 1 e, f, black arrows), confirming that basal cells were actively rearranging β 1 integrin under continual force.

To farther evaluate epidermal responses to TE, we calculated the ratio of basal to apical length for each model (N 3 section/model; 15 images/section) (Fig. 2 a). These data

revealed progressively increased basal layer length with a maximum increase of $71\pm 10\%$ at TE-3d as compared to the control ($p<0.0001$). The comparison of TE-7d ($35\pm 8\%$) and TE-7+7d ($39\pm 4\%$) showed no statistically significant difference, suggesting that the elongation of basal layer slows down considerably in the second week of TE. Basal cell density, calculated as the number of nuclei per $100\ \mu\text{m}$ of basal layer, was initially unchanged between control and treatment (TE-1h and TE-24h), but increased by $9\pm 3\%$ in TE-3d ($p=0.01$) and by $15\pm 2\%$ in TE-7d ($p<0.0001$), indicating that initial stretching favored symmetrical cell divisions (Fig. 2 b). By the 14th day of TE, cell density had normalized to the control level, reaching on average 16 nuclei per $100\ \mu\text{m}$ of basal layer.

Morphological examination of histological sections indicated that the epidermis became more undulated under TE. Quantification of this phenomenon (N 3 sections/model; N 10 images per section) determined that the number of RRs doubled by TE-3d as compared to control ($p<0.0001$) and remained unchanged between TE-7d and TE-7+7d (Fig. 2 c). The surface area of RR (measured on histological sections as illustrated at Fig. 2 d), increased by $33\pm 13\%$ in Ex-7d and $62\pm 19\%$ in TE-7+7d, showing a significant enlargement of RRs in the second week of TE. Growth of the stratified epidermis, calculated as the surface area of the epidermis without RRs, increased by $19\pm 5\%$ (Ex-7d) and $58\pm 7\%$ (TE-7+7d). Total epidermal thickness increased as well, by $44\pm 4\%$ in TE-7d ($p<0.0001$) and by $77\pm 6\%$ in TE-7+7d ($p<0.0001$), as compared to the corresponding controls.

To correlate the above results with cell proliferation, we calculated the mitotic index for each model using IHC with Ki-67 marker (N 3 sections/model; 25 images per section) (Fig. 2 d–f). Relative to controls, mitotic indexes were increased by: $2.8\pm 1\%$ ($p=0.004$) TE-1h, $5.1\pm 1.6\%$ ($p=0.003$) TE-24h, $5.3\pm 1.3\%$ ($p=0.0004$) TE-3d, $5.6\pm 1\%$ ($p<0.0001$) TE-7d, and $5.7\pm 1.1\%$ ($p<0.0001$) for TE-7+7d. Thus, the highest increase in cell proliferation as a result of TE took place within the first 24 hours. Sporadic suprabasal location of Ki-67 and $\beta 1$ integrin positive cells, indicative of local hyperproliferation, was observed in TE-3d at the expansion site over the ribs (Fig. 1 d, blue arrow, and data not shown). Based on our previous work, we expect that these expanders exert higher mechanical stimuli than those placed over the abdomen, suggesting that stretching in this location was in the upper limits of epidermal tolerance to maintain overall healthy tissue.

As basal precursors are intimately connected to BM (Brakebusch et al., 2000; Raghavan, Bauer, Mundscha, Li, & Fuchs, 2000; Yang et al., 2016), we employed Periodic Acid-Schiff (PAS) REF staining to visualize the BM structure (Fig. 2 c - e). While stretches of disorganized BM and thickening areas were found, especially around growing RRs (black arrows), the BM was never obliterated.

Here, we present the effects of acute TE on epidermal structure and basal cells qualities. We found $\beta 1$ integrin to be a very sensitive responder to mechanical stimulation instigated by TE procedure, able to dynamically relocate under external force. The basal cell is naturally polarized by attachment to the BM, and its location along the undulated basal layer dictates the cell's orientation toward the epidermal surface. Thus, cells located at the slope of a RR as compared to those positioned parallel to the epidermal surface are exposed to different force trajectories upon the TE, so they shift $\beta 1$ integrin differently. Based on literature and

our data, we assume that $\beta 1$ integrin accumulated at the lateral cortex of mitotic cells (stripes between cells in TE-1h), indicates the parallel orientation of the mitotic spindle, which favors symmetric cell divisions (Fink et al., 2011; Lechler & Fuchs, 2005; Petridou & Skourides, 2016). The apical location of $\beta 1$ integrin indicates reverse cell polarity and likely impaired asymmetrical division further supports elongation of basal layer. However, we assume that the balance between these two types of cell division was promptly restored because in the second week of TE the accumulation of $\beta 1$ integrin acquired a diffused pattern and this coincided with slower elongation of the basal layer. The expanded epidermis maintained stratified organization through all tested TE procedures, which further indicates the presence of asymmetric divisions required in epidermal homeostasis (Lechler & Fuchs, 2005).

In summary, morphogenetic changes initiated by the relocation of $\beta 1$ integrin represent an adaptive response of the epidermis to mechanical stress. The basal layer elongation and RR growth absorbed the impact of the rapid proliferation in order to preserve the stratified organization and functionality of the tissue. Repeated mechanical stimulation with a seven-day interval generated healthy tissue without detrimental effects, suggesting a good approach for clinical settings.

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Abbreviations:

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| BM | basement membrane |
| IFE | interfollicular epidermis |
| IHC | immunohistochemistry |
| KCs | keratinocytes |
| RRs | rete ridges |
| TE | tissue expansion |

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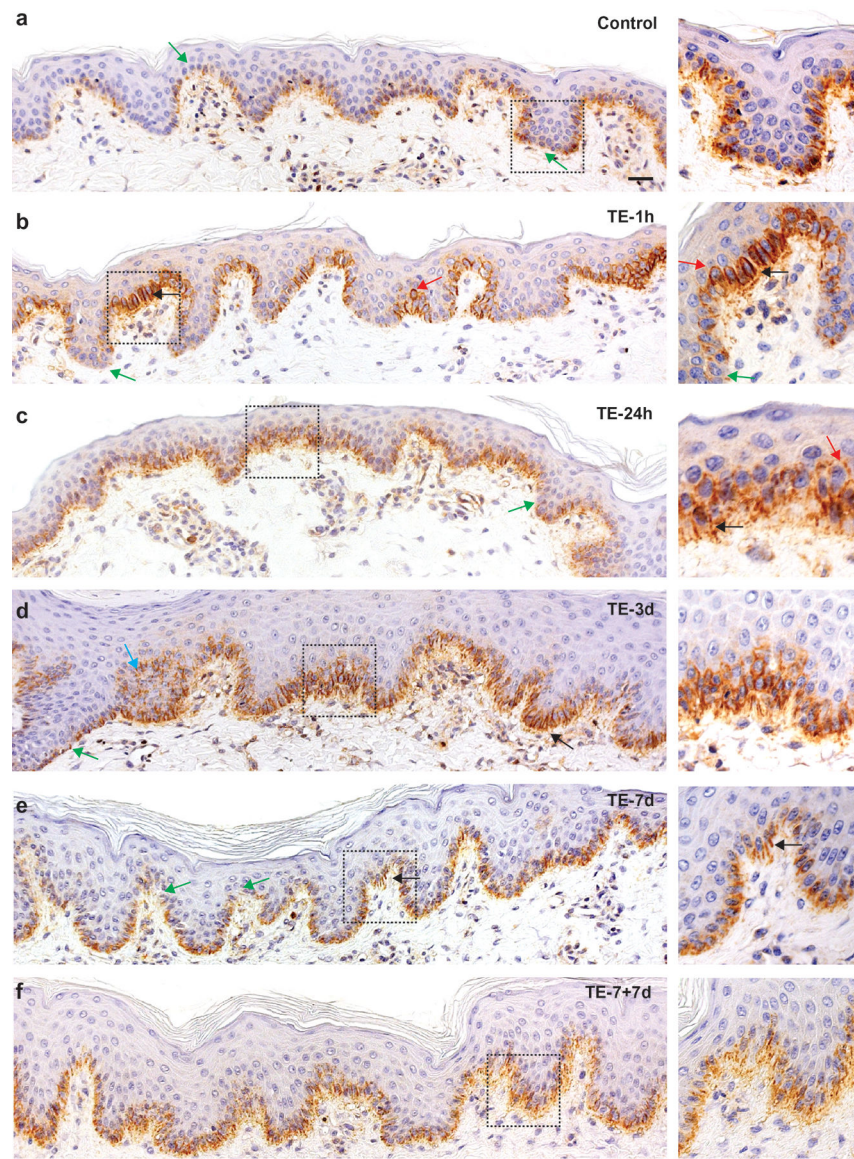


Fig. 1. Changes in localization of $\beta 1$ integrin induced in TE. Representative images of epidermis stained by IHC, nuclear counterstain with hematoxylin; the right panel represents electronically zoomed dashed squares; arrows indicate: green – negative/reduced $\beta 1$ integrin, red – apical localization, black – stripes between adjacent cells, blue - suprabasal location; 20x magnification, scale bar = 20 μm . Images were captured using Axiovision Zeiss microscope and processed in Photoshop.

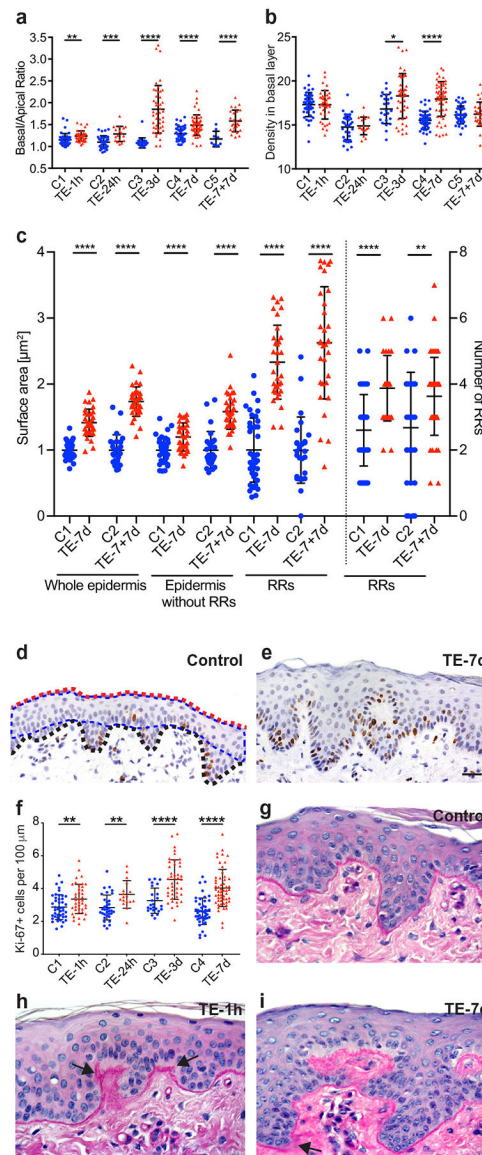


Fig. 2. Quantification of morphological changes in epidermis under TE. (a) The basal/apical length ratio. (b) Cell density calculated as all nuclei per 100 μm of the basal layer. (c) Comparison of the surface area presented as a mean \pm SD and number of RRs between TE-7d and TE-7+7d models. The mean of surface area for C1 was arbitrarily assigned as 1. (d, e) Representative images of IHC for Ki-67 and (f) number of Ki-67 positive cells per 100 μm of the basal layer. The red and black dotted lines indicate apical and basal layers, respectively. The blue dotted line indicates the surface area without RRs. (g-i) The representative images of PAS staining for BM. The measurements were collected and analyzed using ZEN Blue Zeiss software, and GraphPad Prism. Statistical significance was assessed by unpaired Student t-test * p 0.05, ** p 0.01, *** p 0.001, **** p <0.0001; 40x magnification, scale bar = 20 μm . A minimum of three tissue sections for each category and ten images for each section were examined for every model.