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2 **Hair follicle stem cell progeny heal blisters while pausing skin development**

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16

17 [†]Deceased in February 2021

18

19 **Conflicts of Interest**

20 The authors declare that no conflicts of interest exist.

21

22

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29

30 **Abstract**

31 Injury in adult tissue generally reactivates developmental programs to foster
32 regeneration, but it is not known whether this paradigm applies to growing tissue. Here,
33 by employing blisters, we show that epidermal wounds heal at the expense of skin
34 development. The regenerated epidermis suppresses the expression of tissue
35 morphogenesis genes accompanied by delayed hair follicle (HF) growth. Lineage
36 tracing experiments, cell proliferation dynamics, and mathematical modeling reveal that
37 the progeny of HF junctional zone stem cells, which undergo a morphological
38 transformation, repair the blisters while not promoting HF development. In contrast, the
39 contribution of interfollicular stem cell progeny to blister healing is small. These findings
40 demonstrate that HF development can be sacrificed for the sake of epidermal wound
41 regeneration. Our study elucidates the key cellular mechanism of wound healing in skin
42 blistering diseases.

43

44 **Keywords**

45 Wnt signaling, epidermal stem cells, epidermolysis bullosa, basement membrane zone

46

47 **Introduction**

48 Tissue responds to injury by transforming its cellular components and extracellular
49 matrix from homeostasis into a regenerative state. Damaged tissue typically reactivates
50 an embryonic gene program in epithelia to accelerate tissue regeneration (Fernandez
51 Vallone *et al*, 2016; Miao *et al*, 2019; Nusse *et al*, 2018; Yui *et al*, 2018). However, it is
52 unknown whether this phenomenon also applies to injuries in developing tissue, in
53 which the embryonic gene expression program is switched on before damage.

54 The epidermis is a stratified epithelium of the skin and is located on the surface of
55 the body, where it serves as a barrier against external stimuli and microorganisms
56 (Natsuga, 2014). Cellular proliferation and differentiation in the epidermal basal layer,
57 where epidermal stem cells (SCs) are present, are fine-tuned to maintain the integrity of
58 the epidermis (Donati & Watt, 2015). The epidermis attaches to the dermis through
59 proteins in the epidermal basement membrane zone (BMZ) (McMillan *et al*, 2003).
60 Epidermal BMZ proteins function as a niche for epidermal SCs (Watt & Fujiwara, 2011),
61 and the loss of these proteins, such as $\alpha 6$ integrin (ITGA6), $\beta 1$ integrin, and collagen
62 XVII (COL17), leads to transient epidermal proliferation (Brakebusch *et al*, 2000;
63 Niculescu *et al*, 2011; Watanabe *et al*, 2017).

64 Skin wounding causes pain and carries a significant risk of bacterial infection. The
65 sources of skin wound healing have been extensively investigated in experimental
66 animals (Dekoninck & Blanpain, 2019; Rognoni & Watt, 2018). Hair follicles (HFs),
67 epidermal appendages, fibroblasts, and immune cells coordinate to heal the wound, and
68 the contribution of each component can vary depending on the assay (Garcin *et al*,

69 2016). Wounding in adult skin induces the expression of genes regulating epidermal
70 development, including SOX11 and SOX4 (Miao *et al.*, 2019).

71 Conventional skin wounding assays have employed full-thickness skin wounds, in
72 which all skin components are removed, including the epidermis, epidermal
73 appendages, dermis, and subcutaneous fat tissue. In contrast to conventional full-
74 thickness skin wounds, epidermal detachment, as exemplified by subepidermal blisters,
75 is distinctive because it does not affect the structures below the epidermis per se. The
76 epidermis is detached from the dermis in several pathological conditions, such as burns
77 (Chetty *et al.*, 1992), congenital defects in epidermal BMZ proteins (epidermolysis
78 bullosa (EB)) (Fine *et al.*, 2014; Vahidnezhad *et al.*, 2019), autoimmunity to these
79 proteins (pemphigoid diseases) (Schmidt & Zillikens, 2013), and severe drug reactions,
80 such as Stevens-Johnson syndrome/toxic epidermal necrolysis (White *et al.*, 2018).
81 Although the cells that contribute to the repair of full-thickness skin wounds have been
82 identified (Aragona *et al.*, 2017; Dekoninck & Blanpain, 2019; Donati *et al.*, 2017;
83 Gonzales & Fuchs, 2017; Ito *et al.*, 2005; Kang *et al.*, 2020; Page *et al.*, 2013; Park *et al.*,
84 2017; Sada *et al.*, 2016), the cellular dynamics of subepidermal blister healing are
85 completely unknown. In addition, full-thickness skin wounding, when applied to
86 developmental skin, is unsuitable for distinguishing tissue regeneration and
87 development. In contrast, blistering injury allows us to monitor both skin regeneration
88 (reepithelization of the epidermis) and morphogenesis (HF development) within the
89 same wound bed.

90 Here, by taking advantage of subepidermal blisters, we explore the effects of injury
91 on developmental tissue. Unexpectedly, blistering injury is found to reduce the

92 expression of tissue morphogenesis genes in the healed epidermis and to direct HFSCs,
93 rather than epidermal SCs, to provide progeny to heal the wound and to suspend HF
94 development.

95

96

97 **Results**

98 **Subepidermal blister formation and its healing process**

99 The suction-blister technique was developed more than a half-century ago to selectively
100 remove the epidermis from the dermis (Kiistala & Mustakallio, 1964, 1967), and it has
101 been utilized to harvest epidermal pieces for transplant to repair human skin defects.
102 We reasoned that suction blisters on neonatal mice, in which the epidermis is removed
103 while HFs, the dermis, and subcutaneous fat tissues are maintained in the wounds,
104 enable us to examine the direct relationship between tissue injury and skin development.
105 Therefore, we applied constant negative pressure to the dorsal skin of C57BL/6 wild-
106 type (WT) neonates to produce subepidermal blisters (postnatal day 1 (P1), **Figure 1A**).
107 Histologically, skin separation occurred at the level of the dermoepidermal junction
108 (DEJ) (**Figure 1B, Figure EV1A-C**). HFs, as shown by alkaline phosphatase (AP)-
109 positive dermal papillae, remained on the dermal side of the blisters (**Figure 1B, Figure**
110 **EV1D**). Dermis and subcutaneous tissues were intact after blistering (**Figure 1B**). $\alpha 6$
111 integrin (ITGA6), a hemidesmosome protein, was seen at the blister roof, whereas type
112 IV collagen (COL4), a major component of the epidermal basement membrane, and
113 laminin 332 (L332) were present at the base of the blister (**Figure 1C, Figure EV1B**). In
114 line with the immunofluorescence data, hemidesmosomes localized on the blister roof
115 and lamina densa (basement membrane) were observed at the blister base by electron
116 microscopy (**Figure 1D, Figure EV1C**), as seen in human suction blisters (Kiistala &
117 Mustakallio, 1967) and their murine counterparts (Krawczyk, 1971).

118 We then characterized the healing processes of the subepidermal blisters. One to
119 two layers of the regenerated epidermis, marked with pan-cytokeratin, were found one

120 day after blister formation (P2, **Figure 1E**, **Figure EV1E**). The regenerated epidermis
121 restored ITGA6 expression at the DEJ (**Figure 1E**, **Figure EV1E**). The shape of the
122 basal keratinocytes in the intact skin was cuboidal or columnar (P2, keratin 14 (K14)-
123 positive cells in the nonlesional area, **Figure 1F**). In contrast, the regenerated
124 keratinocytes in the blistered skin transformed from cuboidal to a wedge/flattened shape
125 (P2, K14-positive cells in the lesional area, **Figure 1F**) (Krawczyk, 1971). Two days
126 after blistering (P3), the stratified epidermal layers were mostly restored but still lacked
127 loricrin-positive granular layers, a hallmark of proper epidermal differentiation, in the
128 lesional area (**Figure 1G**). The final step in epidermal differentiation was completed by
129 the formation of loricrin-positive granular layers three days after blister formation (P4,
130 **Figure 1H**). The immunofluorescence data for subepidermal healing are summarized in
131 **Table EV1**. These results demonstrate that subepidermal blisters on neonates can
132 serve a model for visualizing wound healing without damaging HFs and other dermal
133 components at the developmental stage.

134

135 **Epidermal restoration at the expense of skin development**

136 To elucidate the effects of the blistering injury on the neonatal skin, we performed RNA-
137 seq profiling of the wounded tissue above the dermis (the regenerated epidermis and
138 the blister roof) one day after blistering (P2, **Figure 2A**, **EV2A**). Unexpectedly, the
139 expression of genes involved in HF morphogenesis, such as Wnt signaling,
140 melanogenesis, and Hedgehog signaling, was significantly downregulated (**Figure 2B**,
141 **2C**, **EV2B-D**, **Dataset EV1**). The HF undergoes morphogenesis in utero and after birth
142 (**Figure EV1F**) (Paus *et al*, 1999; Saxena *et al*, 2019). In murine skin, HF

143 morphogenesis is classified into nine stages: the accumulation of nuclei in the epidermis
144 without downward growth of HFs (stage 0), HFs with the most proximal part in the
145 dermis (stages 1-5), and HFs with the most proximal part in the subcutaneous tissue
146 (stages 6-8) (Paus *et al.*, 1999). The downregulation of HF morphogenesis genes
147 (**Figure 2B, 2C**) led us to hypothesize that epidermal wounding tunes down tissue
148 development to accelerate blister healing. In agreement with this hypothesis, the
149 number of hair canals, which are tube-like connections between the epidermal surface
150 and the most distal part of the inner root sheath (IRS) and are present in only developed
151 HFs (HF morphogenesis, stage 6-8) (Paus *et al.*, 1999), was reduced in the
152 regenerated epidermis (**Figure 2D, 2E**). HF growth under the regenerated epidermis at
153 P4 was delayed at stages 5 and 6 where the IRS is halfway up to the HF or contains the
154 hair shaft up to the level of the hair canal. In contrast, the surrounding intact skin of the
155 blisters or the normal skin of the littermate controls had stage 7 HFs, in which the tip of
156 the hair shaft leaves the IRS and enters the hair canal (**Figure 2F**). The observation of
157 smaller HFs in the skin lesion when compared to the surrounding intact skin is
158 accompanied by diminished Wnt signalling indicated by the LacZ-positive area in Wnt
159 reporter mice (ins-Topgal+) (P2, **Figure 2G**).

160 The expression of genes involved in cytokine-cytokine receptor interactions and
161 chemokine, TNF, IL-17, and JAK-STAT signaling pathways was increased in the
162 regenerated epidermis (**Figure 2B, 2C, EV2B**), and these pathways are implicated in
163 the recruitment of immune cells. However, the number of neutrophils, lymphocytes, and
164 macrophages was not increased in the lesional dermis (P2, **Figure EV1G**), in which 1-2
165 layers of the regenerated epidermis covered the wound one day after blistering. There

166 was no apparent increase of these immune cells either at P4 (**Figure EV1G**), in which
167 the whole epidermis was restored. These results suggest that the immune cells might
168 not play a significant role in blister healing, although the involvement of immune cells or
169 of molecules that they secrete—such as $\gamma\delta$ T cell-derived Fgf9, which induces HF
170 neogenesis (Gay *et al*, 2013)—cannot be fully excluded or may serve as a confounding
171 factor affecting HF growth.

172 These data indicate that, in the context of skin morphogenesis where the immune
173 system is not yet fully defined, subepidermal blisters heal at the expense of HF growth.

174

175 **Progeny of junctional zone SCs represent the main cellular contribution to blister** 176 **healing**

177 Wounded lesions require epithelial cell proliferation and migration to restore skin
178 integrity. We then investigated the dynamics of epidermal and HF keratinocytes during
179 blister healing. One day after blister formation (P2), BrdU⁺ cells were abundant in the
180 HFs and the intact epidermis adjacent to the blisters (**Figure 3A**). Cells positive for $\alpha 5$
181 integrin (ITGA5), a marker of migrating keratinocytes (Aragona *et al.*, 2017), were seen
182 in the HFs within the lesional area and in the epidermal boundary between the blister
183 and the nonlesional area (epidermal tongue) (**Figure 3B, Figure EV1E**). As HF growth
184 was delayed in the regenerated epidermis (**Figure 2D-G**) and proliferative cells were
185 abundant in HFs of the lesional area (**Figure 3A**), HF keratinocytes were deduced to
186 participate in epidermal regeneration rather than in HF development.

187 To confirm this hypothesis, we employed a short-term lineage tracing strategy with
188 suction blistering (**Figure 3C**). K14-lineage labeled cells (K14CreER:R26R-H2B-

189 mCherry or K14CreER:R26R-confetti), mainly progeny of SCs in the interfollicular
190 epidermis (IFE), were sparse in the regenerated epidermis (**Figure 3D, 3E, Figure**
191 **EV3A**). In contrast, most of the cells in the regenerated epidermis were Lrig1 (leucine-
192 rich repeat and immunoglobulin-like domain protein 1)-lineage labeled cells
193 (Lrig1CreER:R26R-H2B-mCherry or Lrig1CreER:R26R-confetti), which are the progeny
194 of junctional zone SCs (**Figure 3F, 3G, Figure EV3A**). In line with this, phospho-
195 Histone H3 (PH3)-positive cells were observed in Lrig1-lineage labeled cells at P2
196 (**Figure EV3B, 3C**). K14- and Lrig1-lineage labeled cells were increased from P1 (at the
197 time of suction blistering) to P4 (sampling), but the expansion of Lrig1-lineage labeled
198 cells was more evident in the regenerated epidermis (**Figure EV3D**). The expression of
199 the Lrig1 gene was not upregulated in the regenerated epidermis at P2 in our RNA-seq.
200 These data indicate that the HF junctional zone on the dermal side of the blister is the
201 main pool for the keratinocytes that heal subepidermal blisters while halting HF
202 development, although other hair follicle populations might also be involved in blister
203 healing.

204

205 **HF reduction from the wound bed of the blisters promotes the contribution of**

206 **IFESC progeny to blister healing**

207 The contribution of junctional zone HFSC progeny to blister healing led us to investigate
208 how the epidermis regenerates in the absence of HFs at the blister base (dermis). Type
209 XVII collagen (COL17) is expressed not only in the IFE but also in the bulge region of
210 the HFs (**Figure 4A**) (Liu *et al*, 2019; Matsumura *et al*, 2016; Natsuga *et al*, 2019;
211 Tanimura *et al*, 2011; Watanabe *et al.*, 2017). COL17 is encoded by the *COL17A1* gene,

212 and its deficiency leads to junctional EB (McGrath *et al*, 1995). The splitting of neonatal
 213 *Col17a1^{-/-}* (Nishie *et al*, 2007) dorsal skin upon suction blistering was observed between
 214 ITGA6 and COL4/L332 (**Figure 4B, 4C**), as was the case in wild-type neonates (**Figure**
 215 **1C, 1D**). Intriguingly, suction blistering (**Figure 1A**) of *Col17a1^{-/-}* dorsal skin detached
 216 most, but not all, of the HFs from the dermis (P1, **Figure 4D**). In agreement with this
 217 finding, dermal papilla cells (AP+) were observed on the roof side of the blisters of
 218 *Col17a1^{-/-}* mice, whereas the blister roofs of control mice did not have these cells (P1,
 219 **Figure 4E, Figure EV1D**). Epidermal regeneration was not apparent in *Col17a1^{-/-}* mice
 220 one day after suction blistering (P2), whereas the control mice showed regeneration of
 221 the epithelial layers (**Figure 4F, Table EV1**). *Col17a1^{-/-}* mice had delayed expression of
 222 loricrin in the regenerated epidermis three days after blister formation (P4, **Figure 4F,**
 223 **Table EV1**). BrdU+ cells were abundant in the *Col17a1^{-/-}* mouse epidermis surrounding
 224 blisters as was the case for controls (**Figure 3A, 4G**). Lineage tracing experiments
 225 (**Figure 3C**) revealed that IFESC progeny covered most of the regenerated area
 226 (K14CreER:R26R-H2B-mCherry:*Col17a1^{-/-}*) at P4 (**Figure 4H, 4I**). The transgenic
 227 rescue of *Col17a1^{-/-}* by overexpressing human COL17 (hCOL17+;*Col17a1^{-/-}*) (Nishie *et*
 228 *al.*, 2007) ameliorated blister healing (P4, **Appendix Figure S1, Table EV1**). These
 229 data demonstrate that upon detachment of most HFs from the dermis, the IFE can
 230 compensate the lack of junctional and HF SCs and repair defects in the IFE.

231

232 **Impaired flattening of regenerated keratinocytes accompanies slower blister**
 233 **healing**

234 We further sought to identify other modulators of subepidermal blister healing. We
235 first focused on collagen VII (COL7), encoded by *Col7a1*. COL7 forms anchoring fibrils
236 and is located at the DEJ (**Figure 5A**) but just below the basement membrane (Shimizu
237 *et al*, 1997; Watanabe *et al*, 2018), and its deficiency leads to dystrophic EB (Christiano
238 *et al*, 1993; Hilal *et al*, 1993). As conventional wound healing is delayed in COL7-
239 hypomorphic mice (Nystrom *et al*, 2013), we applied the suction-blister method to
240 *Col7a1^{-/-}* mice (Heinonen *et al*, 1999) (**Figure 5B-H, Appendix Figure S2**). In contrast
241 to that in WT and *Col17a1^{-/-}* suction blisters (**Figure 1C, 1D, 4B, 4C**), skin splitting
242 occurred at the level below the basement membrane in *Col7a1^{-/-}* mouse dorsal skin, as
243 shown by the presence of L332 and COL4 on the blister roof epidermis (P1, **Figure 5B,**
244 **5C**). The epidermal defects were not repaired in *Col7a1^{-/-}* mice, whereas the epidermis
245 of the control blistered skin regenerated one day after blistering (P2, **Figure 5F,**
246 **Appendix Figure S2, Table EV1**), which is consistent with the delayed healing of full-
247 thickness skin wounds in COL7-hypomorphic mice (Nystrom *et al.*, 2013). This finding is
248 contrasted by the fact that COL7-depleted keratinocytes migrate faster than WT
249 keratinocytes in vitro (Chen *et al*, 2002; Chen *et al*, 2000). We examined the HFs of
250 *Col7a1^{-/-}* mice to explain the slowed epidermal regeneration because HFs are the main
251 contributor to blister healing (**Figure 3A-G**). However, HFs were present in the *Col7a1^{-/-}*
252 mouse wound bed (blister base) (P1, **Figure 5D, 5E**) as opposed to that of *Col17a1^{-/-}*
253 mouse (P1, **Figure 4D, 4E**). Moreover, the number of BrdU+ cells in HFs was
254 comparable between *Col7a1^{-/-}* and control mice (P2, **Figure 5G, Appendix Figure S2**),
255 suggesting that the proliferation of HF keratinocytes does not account for the delayed
256 blister healing of *Col7a1^{-/-}* mice.

257 Second, we treated the blistered skin in wild-type mice with extracellular calcium
258 **(Figure 5I-K, Appendix Figure S2)**. Extracellular calcium is a potent inhibitor of
259 proliferation and migration in cultured keratinocytes as well as an inducer of
260 differentiation (Hennings *et al*, 1980; Magee *et al*, 1987). Consistent with previous in
261 vitro assays, the intrablister administration of CaCl₂ (1.8 mM or 9.0 mM) just after
262 suction blistering delayed epidermal regeneration in vivo (P2, **Figure 5I, Table EV1**).
263 Premature differentiation, which might hinder wound healing, was not apparent in the
264 CaCl₂-treated blisters, as K10 labeling was seen only at the blister roof but not in the
265 keratinocytes on the wound bed (P2, **Figure 5I**). Similar to that in *Col7a1^{-/-}* mouse
266 blisters, the number of BrdU+ cells in HFs was not reduced in CaCl₂-treated blisters (P2,
267 **Figure 5J, Appendix Figure 2**).

268 These two examples strongly suggest that there are factors other than HF
269 keratinocyte proliferation that modulate blister healing. During blister healing,
270 keratinocytes reshape into a wedge-shaped morphology (**Figure 1F**), which is mirrored
271 by the RNA-seq data showing that the expression of genes involved in the regulation of
272 the actin cytoskeleton is decreased in the regenerated epidermis (**Figure 2B, 2C**).
273 Wedge-shaped/flattened keratinocytes are believed to be superior to cuboidal/columnar
274 keratinocytes for covering epidermal defects. These data led us to wonder if the cell
275 morphology was altered in settings of delayed blister healing. In the intact (nonblistered)
276 skin, the morphology of *Col7a1^{-/-}* or Ca-treated basal keratinocytes was similar to that of
277 control cells (P2, the nonlesional area in **Figure 5F, 5H, 5I, 5K**). The regenerated
278 keratinocytes became wedge-shaped/flattened in the control group, as shown in **Figure**
279 **1F**. However, the keratinocytes in the regenerated epidermis of *Col7a1^{-/-}* or Ca-treated

280 mice were not as flat as those of control mice but were still rather cuboidal (P2, the
281 lesional area in **Figure 5F, 5H, 5I, 5K**), which correlates with the delayed blister healing
282 in these mice.

283

284 **Mathematical modeling reproduces blister healing**

285 These in vivo experiments led us to speculate that HF/IFE cell proliferation during
286 paused HF development and the morphological changes of the regenerated
287 keratinocytes might simply account for the dynamics of subepidermal blister healing. To
288 answer this question, we employed mathematical modeling. We adopted an agent-
289 based model (Kobayashi *et al*, 2016; Kobayashi *et al*, 2018), where keratinocytes were
290 modeled by spheroids and cell division was described as replication of the spheroids.
291 Such a model allowed us to visualize the dynamics of the epidermal basal layer and to
292 establish the epidermal defects on the basement membrane. We utilized the data on the
293 number of BrdU+ cells among HF vs. IFE keratinocytes (approximately 7:1 per unit of
294 epidermal length; **Figure 3A**) and on the shape of the regenerated vs. normal
295 keratinocytes (2:1 the length of the major cell axis; **Figure 5H, 5K**). SC progeny within
296 the epidermal defects (colored in red), simulating HF-derived cells, had a more
297 substantial contribution to wound healing than IFE SCs (colored in yellow; **Figure 6A,**
298 **6B, Movie EV1**), as seen in the lineage tracing experiments (**Figure 3C-G**). The
299 absence of SCs within the epidermal defects, simulating HF reduction in the wound bed,
300 showed delayed healing (**Figure 6C, 6D, Movie EV2**), in agreement with *Col17a1*^{-/-}
301 epidermal regeneration results (**Figure 4E-G**). A less flattened morphology of the
302 regenerated keratinocytes, simulating *Col17a1*^{-/-} and Ca-treated blister healing

303 (regenerated vs. normal keratinocytes, 1.3-1.6:1 in length; **Figure 5H, 5K**), slowed
304 epidermal regeneration (**Figure 6E, 6F, Movie EV3**). By systematically changing the
305 shape of regenerated vs. normal keratinocytes (from 1:1 to 2:1 in length) for different
306 initial SC distributions within epidermal defects (**Figure EV4A**), we observed the same
307 tendency: Less flattened morphology led to more delayed healing (**Figure EV4B, EV4C**),
308 which suggests that the morphology affects healing dynamics irrespective of the initial
309 SC distribution. These in silico data demonstrate that the contribution of HFSC progeny
310 and the morphological change in the regenerated keratinocytes are sufficient to
311 recapitulate the in vivo subepidermal blister healing.

312 We finally asked whether this HF contribution to wound healing could be applied to a
313 human setting, in which HFs are larger but much more sparsely distributed than their
314 murine counterparts (**Table EV2**). We examined human subepidermal blister samples
315 and found that epidermal regeneration by HFs was observed in the samples with the re-
316 epithelized area (**Figure EV5**). These findings from human samples are consistent with
317 the mice data.

318 Discussion

319 Although recent studies have reported that injury causes damaged tissue to shift to an
320 embryonic-like state, there is a poor understanding of how regeneration affects
321 development at the damaged tissues. Here, we applied the blistering injury to neonatal
322 mouse dorsal skin and showed the skewed contribution of HFSC progeny to wound
323 healing rather than HF development.

324 Previous studies on skin wounding combined with the fate mapping of murine skin
325 delineated the involvement of epithelial, mesenchymal, and immune cells in wound
326 healing, depending on different settings (Dekoninck & Blanpain, 2019; Rognoni & Watt,
327 2018). However, as full-thickness skin wounds, even when they are applied to neonatal
328 skin, remove all skin components, it is challenging to see the effects of development on
329 injury or vice versa. Our blistering injury has an advantage over conventional skin
330 wounding studies in that only the epidermis is removed by constant negative pressure,
331 with the other skin components and basement membrane being retained in the wounds,
332 which allowed us to examine “pure” epidermal wound healing processes during skin
333 development. Our study contrasts with wound-induced embryonic gene expression
334 (Miao *et al.*, 2019) and follicular neogenesis upon full-thickness skin wounding in adult
335 (Ito *et al.*, 2007; Osaka *et al.*, 2007) and neonatal mice (Rognoni *et al.*, 2016). So far, our
336 study has not distinguished whether the waning Wnt signaling was the primary cause,
337 or the result, of the delayed HF morphogenesis. Further studies are needed to clarify
338 this.

339 Previous studies have suggested the possible involvement of HFs in epidermal
340 regeneration in human suction blisters (Lane *et al.*, 1991) and extracellular matrix

341 alterations in the skin-split area (Hertle *et al*, 1992; Leivo *et al*, 2000). We show that the
342 progeny of HF junctional zone SCs mainly repair subepidermal blisters (**Figure 3F, 3G,**
343 **6A, 6B**), although the involvement of other HF populations cannot be excluded.
344 However, the IFE can also serve as a reservoir of keratinocytes to repair epidermal
345 defects when most HFs are detached from the dermis (Figure 4H). How the
346 contribution of two sources of keratinocytes to blister healing is regulated is unknown,
347 but the significant contribution of HF junctional zone progeny is reasonable because
348 HFs are densely located in the wound bed (blister base). In contrast, the progeny of
349 IFESCs could recover only from the blister edge, as demonstrated by mathematical
350 modeling (**Figure 3D-3G, 6A, 6B**). The role of HFSCs is also highlighted by delayed HF
351 growth in the regenerated epidermis, as corroborated by the downregulated Wnt
352 signaling (**Figure 2A-2G**). These findings indicate that there is a coordinated balance
353 between tissue development and wound healing, which has not been well recognized.
354 In addition, the expression of IL-17 signaling pathway genes was increased, although
355 the recruitment of immune cells was not evident one day or three days after blistering
356 (**Figure EV1G**). Recently, IL-17 signaling has been shown to drive Lrig1-lineage cell
357 recruitment in wound healing and tumorigenesis (Chen *et al*, 2019). Therefore, IL-17
358 signaling might also help Lrig1-lineage cells translocate from HFs to repair epidermal
359 defects in our study (**Figure 3F, 3G**).

360 The dynamics of cytoskeletal changes directly affect cellular morphology and
361 migration potential (Tang & Gerlach, 2017). Previous studies have shown that cells
362 undergo a morphological transformation into a wedge/flattened shape at the leading
363 edge of migrating cells (Uroz *et al*, 2019) and in regenerated keratinocytes during

364 wound healing (Krawczyk, 1971; Paladini *et al*, 1996). Our study has shed further light
365 on the significant impact of the keratinocyte morphological changes on in vivo wound
366 healing through blistering experiments in *Col7a1^{-/-}* and Ca-treated mice and
367 mathematical modeling (**Figure 5F, 5H, 5I, 5K, 6A, 6E, 6F**). However, the
368 morphological changes of keratinocytes might not directly foster blister healing but
369 might be simply correlated with other primary causes that help regenerate the epidermis.
370 The loss of a functional basement membrane in the *Col7a1^{-/-}* blister bottom might slow
371 blister healing due to the lack of substrates for cell migration. Pressure during blister
372 induction could be a factor that changes the cellular morphology. Additional mechanistic
373 studies are needed to verify the hypothesis we raise in our study.

374 Our in vivo blistering experiments can mimic and replace the in vitro cultured cell
375 wound healing assay (e.g., scratch wounding), which has been used in the field of cell
376 biology for decades because subepidermal blisters are epidermal wounds. In vivo
377 intrablister administration of drugs, as exemplified by extracellular calcium
378 administration (**Figure 5I**), could be an alternative to in vitro chemical treatment of
379 scratch-wounded cultured cells to develop new therapeutic options for wound healing,
380 especially of subepidermal blisters.

381 Our in vivo suction-blister model recapitulates the human pathological epidermal
382 detachment seen in EB, pemphigoid diseases, burns, and severe drug reactions such
383 as Stevens-Johnson syndrome/toxic epidermal necrolysis. Loss-of-function mutations in
384 *COL17A1* (McGrath *et al.*, 1995) and *COL7A1* (Christiano *et al.*, 1993; Hilal *et al.*, 1993)
385 lead to junctional and recessive dystrophic EB in humans, respectively. Therefore,
386 *Col17a1^{-/-}* and *Col7a1^{-/-}* mouse blistering also serves as an EB wound model. The

387 prominent hair loss in human *COL17A1*-mutated junctional EB might be reflected by the
388 reduction of HFs from the wound bed in *Col17a1^{-/-}* blisters (**Figure 4D, 4E**), whereas the
389 hair loss in recessive dystrophic EB is not as severe as that in the junctional subtype
390 (Tosti *et al*, 2010), consistent with the maintenance of HFs in the dermis of *Col17a1^{-/-}*
391 mouse dorsal skin upon suction blistering (**Figure 5D, 5E**). It is plausible that recurrent
392 blistering can exhaust the pool of HFSCs, leading to delayed blister healing and
393 scarring, especially in recessive dystrophic EB. Taken together, the processes of
394 subepidermal blister healing highlight HFs as a target for treating the wounds of EB and
395 other blistering diseases.

396 Our study has some limitations, primarily due to the discrepancies between mice
397 and humans. First, eccrine sweat glands have been reported to contribute to wound
398 healing in human skin (Rittie *et al*, 2013). As murine back skin does not harbor sweat
399 glands, our study was unable to estimate the contribution of the sweat glands in human
400 settings. Second, although the skin-split level of human recessive dystrophic EB is
401 generally just beneath lamina densa (basement membrane), as was the case for the
402 *Col17a1^{-/-}* blisters in our study, suction blistering on human recessive dystrophic EB
403 induces skin detachment in the lamina lucida, that is, between hemidesmosomes and
404 the lamina densa (Tidman & Eady, 1984). In addition, our study did not look into the
405 contribution of mesenchymal cells to blister healing, which has been described in
406 previous studies on epidermolysis bullosa (Chino *et al*, 2008; Fujita *et al*, 2010; Inuma
407 *et al*, 2015; Tamai *et al*, 2011; Tolar *et al*, 2009; Webber *et al*, 2017). Further studies
408 are warranted to elucidate the role of mesenchymal cells in blister healing.

409 In closing, our study has revealed the imbalance between development and wound
410 regeneration in the skin blisters. Our findings of the healing processes pave the way for
411 tailored therapeutic interventions for epidermolysis bullosa, pemphigoid diseases and
412 other blistering diseases.

413 **Material and Methods**

414 **Animals**

415 C57BL/6 strain mice were purchased from Clea (Tokyo, Japan). Ins-Topgal+ mice
416 were obtained from RIKEN BRC (Tsukuba, Japan) (Moriyama *et al*, 2007).
417 K14CreER, Lrig1CreER, and R26R-confetti mice were purchased from the
418 Jackson Laboratory (Bar Harbor, Maine, USA). R26R-H2B-mCherry mice were
419 provided by RIKEN (Kobe, Japan). *Col17a1^{-/-}* and *hCOL17+;Col17a1^{-/-}* mice were
420 generated as previously described (Nishie *et al.*, 2007). *Col7a1^{-/-}* mice were
421 provided by Prof. Jouni Uitto (Heinonen *et al.*, 1999). The institutional review board
422 of the Hokkaido University Graduate School of Medicine approved all animal
423 studies described below.

424

425 **Suction blisters**

426 Suction blisters were produced on the neonatal murine dorsal skin (P1) using a syringe
427 and connector tubes. The negative pressure applied to the skin (generally for minutes)
428 was 523.4 ± 1.3 mmHg (evaluated by an Ex Pocket Pressure Indicator PM-281 (AS ONE,
429 Osaka, Japan)). The diameter of the syringe attached to the skin was 4 mm. The size of
430 the typical blister was 3 mm in diameter.

431

432 **Histology**

433 Mouse dorsal skin specimens were fixed in formalin and embedded in paraffin after
434 dehydration or were frozen on dry ice in an optimal cutting temperature (OCT)
435 compound. Frozen sections were fixed with 4% paraformaldehyde (PFA) or cold

436 acetone or were stained without fixation. Antigen retrieval with pH 6.0 (citrate) or pH 9.0
437 (EDTA) buffer was performed on deparaffinized sections. Sections were incubated with
438 primary antibodies overnight at 4°C. After being washed in phosphate-buffered saline
439 (PBS), the sections were incubated with secondary antibodies conjugated to FITC,
440 Alexa 488, Alexa 647 or Alexa 680 for 1 hr at room temperature (RT). The nuclei were
441 stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI). The stained
442 immunofluorescent samples were observed using a confocal laser scanning microscope
443 (FV-1000 (Olympus, Tokyo, Japan) or LSM-710 (Zeiss, Oberkochen, Germany)).

444 For immunohistochemistry, horseradish peroxidase (HRP)-tagged secondary
445 antibodies were used. Sections were blocked with hydrogen peroxide, labeled with
446 antibodies, and counterstained with hematoxylin. For morphological analysis,
447 deparaffinized sections were stained with hematoxylin and eosin (H&E) by conventional
448 methods. Alkaline phosphatase staining was performed using a StemAb Alkaline
449 Phosphatase Staining Kit II (Stemgent, San Diego, California, USA). Images of
450 immunohistochemistry, and H&E- and alkaline phosphatase-stained sections were
451 captured with a BZ-9000 microscope (Keyence, Tokyo, Japan).

452 For whole-mount staining, mouse dorsal skin samples were fixed with 4% PFA and
453 immunolabeled or stained with the Alkaline Phosphatase Staining Kit II. For X-gal
454 staining of ins-Topgal+ mouse skin, a beta-galactosidase staining kit (Takara-bio, Shiga,
455 Japan) was used according to the provider's protocol. Briefly, dorsal skin samples were
456 fixed with 4% PFA for 1 hr at 4°C and soaked in staining solution overnight at RT.
457 Tissues were mounted in a Mowiol solution. Images were observed with LSM-710, FV-
458 1000 or BZ-9000 microscopes.

459 HF morphological stages were evaluated as previously described (Paus *et al.*, 1999).
460 The length of the major axis of keratinocytes in the intact and regenerated epidermis
461 was measured using ImageJ (NIH, Bethesda, Maryland, USA) on K14-stained sections.
462 The quantification of the cells expressing a particular marker was performed as
463 previously described (Natsuga *et al*, 2016).

464

465 **Antibodies**

466 The following antibodies were used: anti-BrdU (Abcam, Cambridge, UK; BU1/75, Dako;
467 M0744), anti-phospho-Histone H3 (Ser10) (Merck Millipore, Billerica, Massachusetts,
468 USA), anti-Ioricrin (Covance, Princeton, New Jersey, USA), FITC-conjugated anti-CD3e
469 (BioLegend, San Diego, California, USA; 145-2C11), Alexa Fluor 488-conjugated anti-
470 F4/80 (Affymetrix, Santa Clara, California, USA; BM8), FITC (fluorescein
471 isothiocyanate)-conjugated anti-Ly-6G (Beckman Coulter, Brea, California, USA; RB6-
472 8C5), anti-COL4 (Novus Biologicals, Centennial, Colorado; NB120-6586), anti-COL7
473 (homemade (Iwata *et al*, 2013)), anti- COL17 (Abcam; ab186415), anti-ITGA5 (Abcam;
474 EPR7854), anti-ITGA6 (BD Biosciences Pharmingen, San Diego, California, USA;
475 GoH3), anti-L332 (Abcam; ab14509), anti-laminin β 1 (Abcam; ab44941), anti-pan-
476 cytokeratin (PROGEN, Wieblingen, Heidelberg, Germany; PRGN-10550), anti-
477 cytokeratin 10 (Biolegend; Poly19054), anti-cytokeratin 14 (ThermoFisher, Waltham,
478 Massachusetts, USA; LL002).

479

480 **BrdU labeling**

481 For proliferation analysis, 10 µg of BrdU (BD Biosciences Pharmingen) per head was
482 intraperitoneally administered 4 hr before sacrifice.

483

484 **Transmission electron microscopy**

485 The samples were taken from C57BL/6 mouse dorsal skin (P1) just after suction
486 blistering was performed. The samples were fixed in 5% glutaraldehyde solution,
487 postfixed in 1% OsO₄, dehydrated, and embedded in Epon 812. The embedded
488 samples were sectioned at 1 µm thickness for light microscopy and thin-sectioned for
489 electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate
490 and lead citrate and examined by transmission electron microscopy (H-7100; Hitachi,
491 Tokyo, Japan).

492

493 **Lineage tracing**

494 K14CreER:R26R-H2B-mCherry, Lrig1CreER:R26R-H2B-mCherry, K14CreER:R26R-
495 confetti, and Lrig1CreER:R26R-confetti mice were intraperitoneally treated with 0.5 mg
496 of tamoxifen (T5648; Sigma-Aldrich, St. Louis, Missouri, USA) at P0. The dorsal skin
497 samples were harvested four days later (P4).

498

499 **RNA sequencing and analysis**

500 Suction-blistered and control samples were collected at P2 at the same time of day to
501 exclude the effects of circadian oscillations on epidermal gene expression (Janich *et al*,
502 2013). The skin samples were treated with 0.25% trypsin EDTA overnight at 4°C. The
503 blistered and regenerated epidermis was collected by separating it from the dermis,

504 minced with a scalpel and suspended in 10% FCS DMEM. The cell suspension was
505 filtered through a 70 µm filter, and cell pellets were collected. Library preparation was
506 performed using an Illumina TruSeq RNA prep kit by following the manufacturer's
507 instructions. Briefly, following TRIzol extraction and chemical fragmentation, mRNA was
508 purified with oligo-dT-attached magnetic beads and reverse transcribed into cDNA.
509 Following a second strand synthesis step with DNA polymerase I and RNase H, the
510 resulting cDNA was subjected to end repair, A-tailing, and Illumina compatible adaptor
511 ligation. Following purification and PCR-mediated enrichment, libraries were purified
512 with AMPure XP beads and sequenced on a NextSeq 500 Illumina sequencer.

513 After quality controls were performed, the raw reads were aligned to the NCBI m37
514 mouse reference genome (mm9) using HiSat2 (Kim *et al*, 2015) (version 2.0.0) using
515 options -N 1 -L 20 -i S, 1, 0.5 -D 25 -R 5 --pen-noncansplice 20 --mp 1, 0 --sp 3, 0 and
516 providing a list of known splice sites. Expression levels were quantified using
517 featureCounts (Liao *et al*, 2014) with RefSeq gene annotation and normalized as TPM
518 using custom scripts. Differential expression analysis was performed using the edgeR
519 (Robinson *et al*, 2010) software package. After lowly expressed genes (1 count per
520 million in less than two samples) were filtered out, the treatment and control groups
521 were compared using the exact test method (Robinson *et al.*, 2010). Genes with an
522 absolute log₂-fold change greater than 1 and false discovery rate (FDR) less than or
523 equal to 0.05 were considered differentially expressed. Hierarchical clustering of gene
524 expression profiles was performed on differentially expressed genes using only
525 Euclidean distances and the complete linkage method. TPM values were normalized as
526 Z-scores across samples, and the distances were computed. GO term, KEGG pathway

527 enrichment analysis on differentially expressed genes and GO term network
528 visualization were performed using the clusterProfiler R/Bioconductor package (Yu *et al*,
529 2012). To validate the enriched pathways, GSEA analysis was performed.

530

531 **Intrablister administration**

532 Ten microliters of 1.8 or 9.0 mM CaCl₂ in PBS was administered by syringe into the
533 blisters just after the suction blistering procedure was performed.

534

535 **Statistics**

536 Statistical analyses were performed using GraphPad Prism (GraphPad Software, La
537 Jolla, California, USA). P-values were determined using Student's t-test or one-way
538 ANOVA followed by Tukey's test. P-values are indicated as *0.01<p<0.05,
539 **0.001<p<0.01, ***0.0001<p<0.001, and ****p<0.0001. The values were shown as the
540 means ± standard errors (SE), violin plots or connected with lines showing individual
541 mice.

542

543 **Mathematical modeling**

544 A mathematical model proposed for epidermal cell dynamics (Kobayashi *et al.*, 2016;
545 Kobayashi *et al.*, 2018) was adapted to simulate epidermal wound healing. (For the
546 detailed mathematical formulation, see the Appendix Supplementary Methods and
547 **Dataset EV2.**) In this model, epidermal basal cells were represented as spherical
548 particles, with the cell diameter set to 10 µm. Cells designated as epidermal SCs and
549 their progeny could undergo division on the basement membrane. Cell division was

550 described as a process of two initially completely overlapping particles gradually
551 separating into two distinct particles. When a newly created cell was not fully
552 surrounded by other cells, it was judged as being in a regeneration process and
553 immediately underwent a transition to an oblate spheroid shape with the long axis
554 increased by a factor of 2 (normal) or 1.5 (simulating *Col7a1*^{-/-} and Ca-treated blisters)
555 while its volume was kept constant. The same division rate was assigned to all
556 proliferative cells, with an average division period of 57.6 [arb. unit]. Forces exerted on a
557 cell came from adhesion and excluded-volume interactions with other cells and with the
558 basement membrane. SCs were tightly bound and unable to detach from the basement
559 membrane, while the progeny were weakly bound so that they could detach from the
560 membrane via the ambient pressure: the detached cells were removed from the system.
561 The basement membrane was assumed to be a rigid flat surface, whose shape
562 remained unchanged over time. These interactions were calculated to obtain the time
563 evolution of the whole system by solving equations given in a previous report
564 (Kobayashi *et al.*, 2018). The simulation region was set to 600 μm x 600 μm horizontally
565 with periodic boundary conditions. To prepare the initial conditions for the simulation of
566 subepidermal blister healing, we first ran a simulation with SCs placed on the basement
567 membrane until their progeny covered the whole surface. Then, we set the progeny to
568 be nonproliferative and created epidermal defects by removing the cells that were inside
569 a disk domain with a diameter of 480 μm .

570

571 **Human samples**

572 From the H&E-stained skin of patients with congenital or autoimmune subepidermal
573 blistering diseases (73 EB or 188 bullous pemphigoid (BP) samples, respectively), the
574 samples that met the following histological criteria were selected: (1) subepidermal
575 blisters or a skin split at the dermoepidermal junction; (2) re-epithelization in the area;
576 and (3) presence of HFs on the blister base. Three BP samples fulfilled all the criteria
577 (blisters 1, 2, and 3) and were observed with a Keyence BZ-9000 microscope. The
578 institutional review board of the Hokkaido University Graduate School of Medicine
579 approved all human studies described above (ID: 13-043 and 15-052). The study was
580 conducted according to the Declaration of Helsinki Principles. Participants or their legal
581 guardians provided written informed consent.

582

583 **Data availability**

584 The datasets produced in this study are available in the following databases:

585

586 - RNA-Seq data: Gene Expression Omnibus GSE154871

587 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154871>)

588

589 **Author contributions**

590 Y. F. designed and performed the experiments, analyzed the data, interpreted the
591 results, and wrote the manuscript. M. W., S. T., H. N., H. K., Y. W., Y. M, A. L., V. P., H.
592 U., H. I., W. N., and S. O. performed the experiments and analyzed the data. K. O., Y.
593 K., and M. N. performed the mathematical modeling and wrote the manuscript. G. D.
594 designed and performed the experiments, analyzed the data and interpreted the results.

595 H. S. interpreted the results and supervised the study. K. N. conceived and designed
596 the experiments, analyzed the data, interpreted the results, wrote the manuscript and
597 supervised the study.

598

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607 died while this manuscript was in revision. We dedicate this paper to him.

608

609 **Conflict of interest**

610 The authors declare that they have no conflict of interest.

611

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839

840 **Figure Legends**841 **Figure 1. Healing of subepidermal blisters in neonatal mice.**

842 **A** Schematic diagram of suction blistering and sample collection. A blister produced on
843 C57BL/6 wild-type (WT) mouse dorsal skin at P1. BM: basement membrane.

844 **B-D** Blistered samples at P1. (B) Hematoxylin and eosin (H&E, top) and alkaline
845 phosphatase (AP, bottom) staining. Hair follicles (HFs) remaining in the dermis
846 (indicated by arrows). Scale bar: 500 μ m. (C) α 6 Integrin (ITGA6, indicated by
847 arrowheads) and type IV collagen (COL4, arrows) labeling (left). Laminin 332 (L332,
848 arrows) staining (right). Scale bar: 100 μ m. (D) Ultrastructural findings of blistered skin
849 (left image: blister roof, right image: blister bottom) . Hemidesmosomes (white
850 arrowheads) and lamina densa (arrows) are indicated. Scale bar: 1 μ m.

851 **E** H&E (left), pan-cytokeratin (PCK, middle), and ITGA6 staining (right) at P2. The
852 regenerated epidermis is indicated by arrowheads. Scale bar: 200 μ m.

853 **F** Keratin 14 (K14) and keratin 10 (K10) staining of the nonlesional (intact) and lesional
854 (blistered) skin at P2 (upper images and inlets: sections, lower images: whole-mount
855 imaging). HFs are indicated by arrows in the whole-mount images. Scale bar: 30 μ m.

856 **G** H&E (top) and loricrin (LOR, bottom) staining at P3. Scale bar: 200 μ m.

857 **H** H&E (left), PCK (middle), and LOR staining (right) at P4. Scale bar: 200 μ m.

858 Data information: Blisters are indicated by stars. Representative images are shown from
859 three or more replicates in each group.

860 **Figure 2. Delayed HF growth during subepidermal blister healing.**

861 **A** Heat map (Pearson's correlation) of differentially expressed genes between the
862 blistered (regenerated) and control WT dorsal skin epidermis at P2 (n=3).

863 **B** GO analysis of differentially expressed genes in the regenerated epidermis.

864 **C** Scatter plots of differentially expressed genes in the regenerated epidermis. The red
865 dots represent upregulated genes, and the blue dots represent downregulated genes.
866 The gray dotted lines indicate $|\logFC|>1$.

867 **D** Hair canals in the regenerated (lesional) and nonlesional epidermis at P4 (indicated
868 by asterisks). Scale bar: 300 μm .

869 **E** Quantification of hair canals in the lesional, nonlesional, and unaffected littermate
870 control epidermis at P4 (n=5 biological replicates). The data are shown as the mean \pm
871 SE (littermate control) or connected with lines showing individual mice. *0.01<p<0.05,
872 one-way ANOVA test, followed by Tukey's test.

873 **F** HF morphogenesis stages at P4 in lesional, nonlesional, and unaffected littermate
874 control skin (n=5 biological replicates).

875 **G** Whole-mount imaging of the blistered skin of ins-Topgal+ mice at P2. Scale bar:
876 500 μm .

877 Data information: Representative images are shown from three or more replicates in
878 each group.

879

880 **Figure 3. Predominant contribution of HF-derived keratinocytes to subepidermal**
881 **blister healing.**

882 **A** (Top) BrdU labeling of blistered samples at P2. Scale bar: 100 μm . BrdU-positive cells
883 are indicated by arrows. Blisters are indicated by stars. (Bottom) Quantification of BrdU-
884 positive cells in the epidermis (left) and HFs (right) (n=4 biological replicates). The data
885 are shown as the mean \pm SE. *0.01<p<0.05, one-way ANOVA test, followed by Tukey's
886 test. NS, no significance.

887 **B** $\alpha 5$ integrin (ITGA5) labeling at P2 (left image: section, right image: whole-mount).
888 Scale bar: 100 μm . Blister edges (epidermal tongue) and HFs are indicated by
889 arrowheads and arrows, respectively. Blisters are indicated by stars.

890 **C** Lineage tracing strategy.

891 **D** (Top) Sections of K14CreER:H2B-mCherry mice at P4. Scale bar: 100 μm . (Bottom)
892 Quantification of mCherry-positive cells (n=3).. The data from individual mice are
893 connected by lines. Student's t-test. NS, no significance.

894 **E** Whole-mount imaging of K14CreER:R26R-confetti samples at P4. Scale bar: 200 μm .

895 **F** (Top) Sections of Lrig1CreER:H2B-mCherry mouse skin at P4. Scale bar: 100 μm .
896 (Bottom) Quantification of mCherry-positive cells (n=3). The data from individual mice
897 are connected by lines. *0.01<p<0.05, Student's t-test.

898 **G** Whole-mount imaging of Lrig1CreER:R26R-confetti mouse samples at P4. Scale bar:
899 200 μm .

900 Data information: Representative images are shown from three or more replicates in
901 each group.

902

903 **Figure 4. Effects of HF reduction on subepidermal blister healing.**

904 **A** Type XVII collagen (COL17, arrowheads indicate the hair bulge) and laminin β 1

905 (LAMB1) labeling in WT dorsal skin sections (P1). Scale bar: 100 μ m.

906 **B, C** Blistered samples of *Col17a1^{-/-}* mouse dorsal skin at P1. ITGA6 (indicated by

907 arrowheads) and COL4 (arrows) labeling (B). L332 staining (C, arrows). Scale bar:

908 100 μ m.

909 **D** H&E staining of blistered skin from *Col17a1^{-/-}* mice at P1. HFs detached from the

910 dermis in *Col17a1^{-/-}* skin are indicated by arrowheads. Scale bar: 500 μ m.

911 **E** Whole-mount AP staining of the blister roof epidermis from *Col17a1^{-/-}* mice (right) and

912 littermate controls (left) at P1. Scale bar: 500 μ m.

913 **F** H&E (P2, left), ITGA6 (P2, middle) and LOR staining (P4, right) of *Col17a1^{-/-}* mice

914 (top) and littermate controls (bottom) . The regenerated epidermis is indicated by

915 arrowheads. Scale bar: 200 μ m.

916 **G** (Top) BrdU labeling of *Col17a1^{-/-}* skin at P2. Scale bar: 100 μ m. (Bottom)

917 Quantification of BrdU-positive cells in the epidermis surrounding blisters (n= 3 (control)

918 and 4 (*Col17a1^{-/-})* biological replicates). The data are shown as the mean \pm SE.

919 Student's t-test. NS, no significance.

920 **H, I** (H) Lineage tracing of K14CreER:R26R-mCherry:*Col17a1^{-/-}* at P4. Scale bar:

921 100 μ m. (I) Quantification of mCherry-positive cells in the regenerated epidermis (n=3

922 biological replicates). The data from individual mice are connected by lines.

923 *0.01<p<0.05, Student's t-test.

924 Data information: Blisters are indicated by stars. Representative images are shown from

925 three or more replicates in each group.

926 **Figure 5. Involvement of keratinocyte shape transformation in subepidermal**
 927 **blister healing.**

928 **A** Type VII collagen (COL7) labeling in WT dorsal skin sections (P1). Scale bar: 200 μ m.

929 **B** ITGA6/COL4 (left, indicated by arrows) and L332 labeling (right, indicated by arrows)
 930 in the blistered skin of *Col7a1^{-/-}* mice at P1. Scale bar: 100 μ m.

931 **C** Schematic diagram of control, *Col17a1^{-/-}*, and *Col7a1^{-/-}* mouse skin splits. BM:
 932 basement membrane.

933 **D** H&E staining of blistered skin from *Col7a1^{-/-}* mice (right) and their littermate controls
 934 (left) at P1. Scale bar: 200 μ m.

935 **E** Whole-mount AP staining of the blister roof epidermis from *Col7a1^{-/-}* mice (right) and
 936 their littermate controls (left) at P1. Scale bar: 500 μ m.

937 **F** K10/K14 (low magnification, left) and K14 (high magnification, middle and right)
 938 labeling of *Col7a1^{-/-}* mouse (bottom) and littermate control (top) blistered skin at P2.
 939 Scale bar: 30 μ m.

940 **G** Quantification of BrdU-positive cells per μ m HF length (n=55 HFs from three control
 941 and 143 HFs from four *Col7a1^{-/-}* mice). The data are shown as violin plots. Student's t-
 942 test. NS, no significance.

943 **H** Length of the major axis of keratinocytes in the regenerated epidermis (n=244 (control,
 944 L), and 132 (*Col7a1^{-/-}*, L) cells from four mice, respectively) and in the surrounding intact
 945 epidermis (basal cells; n=200 (control, NL), 299 (*Col7a1^{-/-}*, NL), from four mice,
 946 respectively). NL: nonlesional area. L: lesional area. The data are shown as violin plots.

947 ****p<0.0001, one-way ANOVA test, followed by Tukey's test. NS, no significance.

948 **I** K10/K14 (left) and K14 (high magnification, right) labeling of WT blistered skin treated
949 with CaCl₂ (middle and bottom) or PBS (top) at P2. Scale bar: 30 μm.

950 **J** Quantification of BrdU-positive cells per μm HF length (n=83 (PBS), 95 (1.8 mM
951 CaCl₂), and 97 (9.0 mM CaCl₂) HFs from four mice). One-way ANOVA test, followed by
952 Tukey's test. NS, no significance.

953 **K** Length of the major axis of keratinocytes in the regenerated epidermis (n=433 (PBS,
954 L), 451 (1.8 mM CaCl₂, L), and 425 (9.0 mM CaCl₂, L) cells from four mice) and in the
955 surrounding intact epidermis (basal cells; n=311 (PBS, NL), 279 (1.8 mM CaCl₂, NL),
956 302 (9.0 mM CaCl₂, NL) cells from four mice). NL: nonlesional area. L: lesional area.

957 The data are shown as violin plots. ****p<0.0001, one-way ANOVA test, followed by
958 Tukey's test. NS, no significance.

959 Data information: Blisters are indicated by stars. Representative images are shown from
960 three or more replicates in each group. The dashed and dotted lines in the violin plots
961 show the median and quartiles, respectively.

962

963 **Figure 6. Mathematical modeling of subepidermal blister healing.**

964 **A** A particle-based model of subepidermal blister healing at the basal layer. Epidermal
965 basal cells (colored in blue), which do not divide, are placed on the basement
966 membrane (gray). Stem cells (SCs, green) give rise to progeny (simulating HF-derived
967 cells; red) within epidermal defects or in the surrounding epidermis (IFE-derived cells;
968 yellow). t : arbitrary time. See **Movie EV1**.

969 **B** Contribution of each progeny cell within the epidermal defect or of surrounding
970 epidermis to subepidermal blister healing, measured as the ratio of the area occupied
971 by each progeny to the area of the initial epidermal defect.

972 **C** A model of subepidermal blister healing without SCs within epidermal defects. See
973 **Movie EV2**.

974 **D** Time course of subepidermal blister healing in control (A) and SC-depleted epidermal
975 defects (C).

976 **E** Effects of the impaired flattening of keratinocytes upon epidermal regeneration. The
977 diameter of basal keratinocytes (long axis of the spheroid) in the regenerated vs.
978 surrounding epidermis was calculated as 1.5:1 (in contrast to 2:1 in Figure 6A). See
979 **Movie EV3**.

980 **F** Time course of wound healing for control (A) and less flattened keratinocytes in the
981 regenerated epidermis (E).

982

983 **Expanded View Figure Legends**

984 **Figure EV1. Healing processes of subepidermal blisters.**

985 **A** H&E (left) and PCK labeling (right) of blistered skin at P1 (WT). Scale bar: 100 μm .

986 **B** ITGA6 (arrowheads in the blister roof) and COL4 (arrows in the blister bottom)

987 labeling at P1 (WT). HFs that express ITGA6 on the dermal side are indicated by

988 hashtags. Scale bar: 100 μm (left) and 50 μm (right).

989 **C** Electron microscopy of blistered skin at P1 (WT). Hemidesmosomes (white

990 arrowheads) and the lamina densa (arrows) are indicated. Scale bar: 10 μm (left) and 1

991 μm (right).

992 **D** AP staining of WT (left) and *Col17a1*^{-/-} (right) blistered skin at P1. Scale bar: 100 μm .

993 **E** H&E (left), ITGA6 (middle), and ITGA5 (right) staining at P2 (WT). The regenerated

994 epidermis is indicated by arrowheads. ITGA5+ cells at the tip of the HFs are indicated

995 by arrows. Blisters are indicated by stars. Scale bar: 100 μm .

996 **F** Schematic of the suction blister experiments and HF development/cycles.

997 **G** Quantification of immune cells (CD3, F4-80, and Ly6G) in the dermis of blistered WT

998 and unaffected littermate control skin at P2 (n=4 biological replicates) and P4 (n=3

999 biological replicates). The data are shown as the mean \pm SE. *0.01<p<0.05, Student's t-

1000 test. NS, no significance.

1001 Data information: Blisters are indicated by stars. Representative images are shown from

1002 three or more replicates in each group.

1003

1004

1005

1006 **Figure EV2. RNA-seq data on subepidermal blister healing.**

1007 **A** Volcano plot showing differentially expressed genes (DEG) between the blistered
1008 (regenerated) and control skin epidermis at P2. Significantly ($|\text{LogFC}| > 1$; $\text{FDR} < 0.05$) up-
1009 regulated and down-regulated DEG are shown in red and blue, respectively.

1010 **B** Bar plot summarizing GSEA enrichment results for selected up-regulated and down-
1011 regulated KEGG pathways. The normalized enrichment score (NES), p-value and FDR
1012 are shown.

1013 **C** GSEA enrichment plots of “Wnt signaling pathway”, “Hedgehog signaling pathway”
1014 and “Melanogenesis” KEGG gene sets.

1015 **D** Network visualization of the top ten down-regulated and up-regulated ($\text{FDR} < 0.05$) GO
1016 term clusters for different GO categories (Biological process, Molecular Function and
1017 Cellular Component). Node size reports the number of enriched genes in each GO term
1018 (gene numbers in bottom panels). Nodes are colored as a pie chart depicting the
1019 proportion of down-regulated (blue) and up-regulated (red) genes in each GO term.
1020 Edge thickness depicts the number of shared genes between GO terms.

1021

1022 **Figure EV3. Lineage tracing of subepidermal blister healing.**

1023 **A** Blistered area of K14CreER:R26R-confetti (left) and Lrig1CreER:R26R-confetti (right)

1024 mouse skin samples at P4. Scale bar: 100 μm .

1025 **B** Phospho-Histone H3 (PH3) staining of blistered skin at P2 (WT). Scale bar: 100 μm .

1026 **C** PH3 staining_(arrowhead) of blistered skin at P2 (Lrig1CreER:R26R-H2BmCherry).

1027 Scale bar: 100 μm .

1028 **D** Quantification of mCherry-positive cells during lineage tracing (n=3 biological

1029 replicates). The data are shown as the mean \pm SE.

1030 Data information: Blisters are indicated by stars. Representative images are shown from

1031 three or more replicates in each group.

1032

1033 **Figure EV4. Dependence of subepidermal blister healing on flattening ratio and**
1034 **SC arrangement.**

1035 **A** Arrangements of the SCs within epidermal defects (dispersed, 2 cells grouped, 4 cells
1036 grouped, and 8 cells grouped) at $t = 0$.

1037 **B** Time course of subepidermal blister healing for different keratinocyte flattening ratios
1038 (1.0-2.0), each with four SC arrangements.

1039 **C** Time to full recovery (100% healing rate) for different keratinocyte flattening ratios,
1040 averaged over four SC arrangements. The data are shown as the mean \pm SD.

1041

1042 **Figure EV5. Subepidermal blister healing in humans.**
1043 H&E staining of human subepidermal blister samples with re-epithelized areas (blisters
1044 1, 2, and 3). Blisters are indicated by stars. The regenerated epidermis from HFs is
1045 indicated by arrows. Scale bar: 300 μm .
1046

Figure 1. Healing of subepidermal blisters in neonatal mice

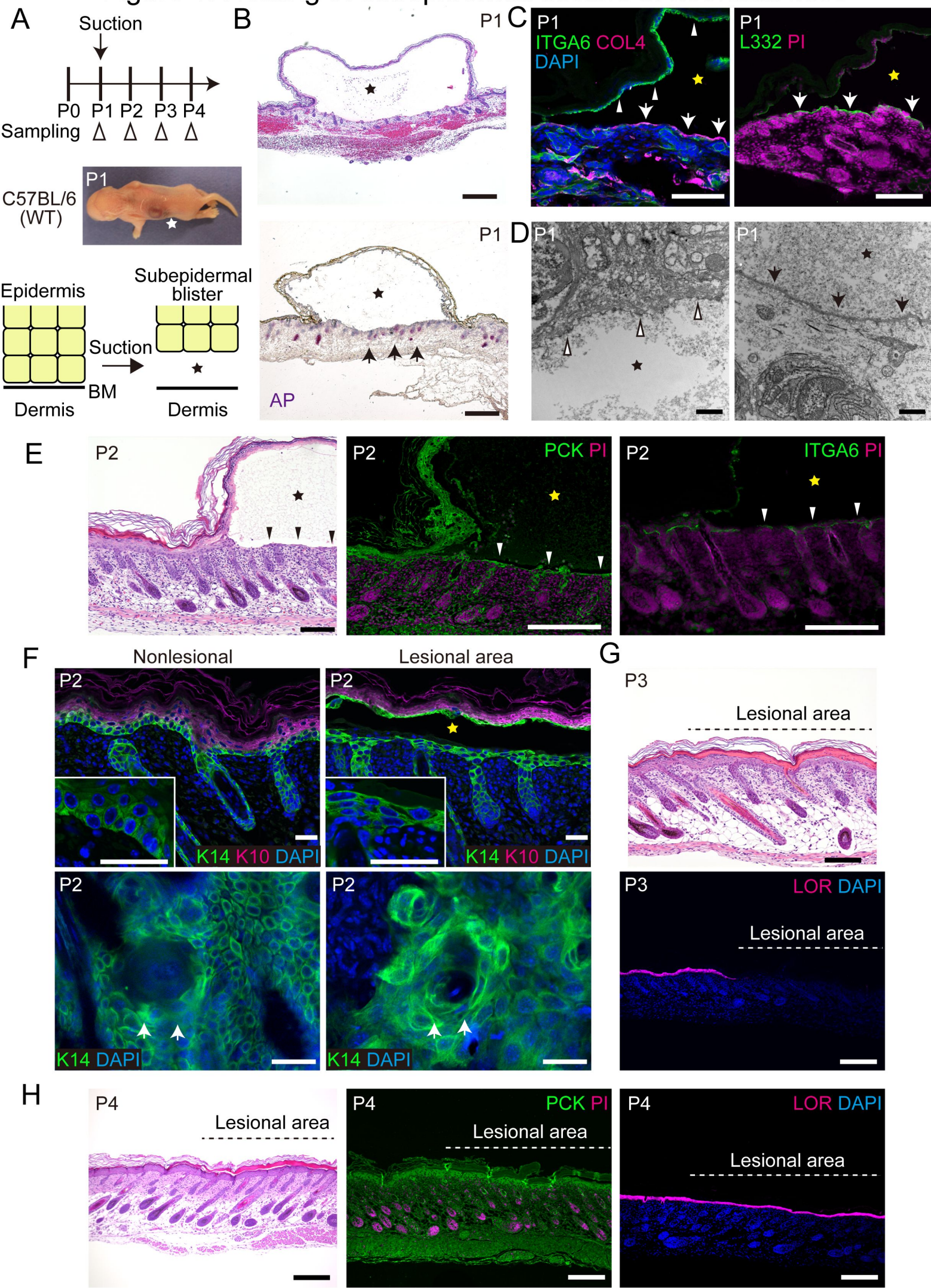


Figure 2. Delayed HF development during subepidermal blister healing

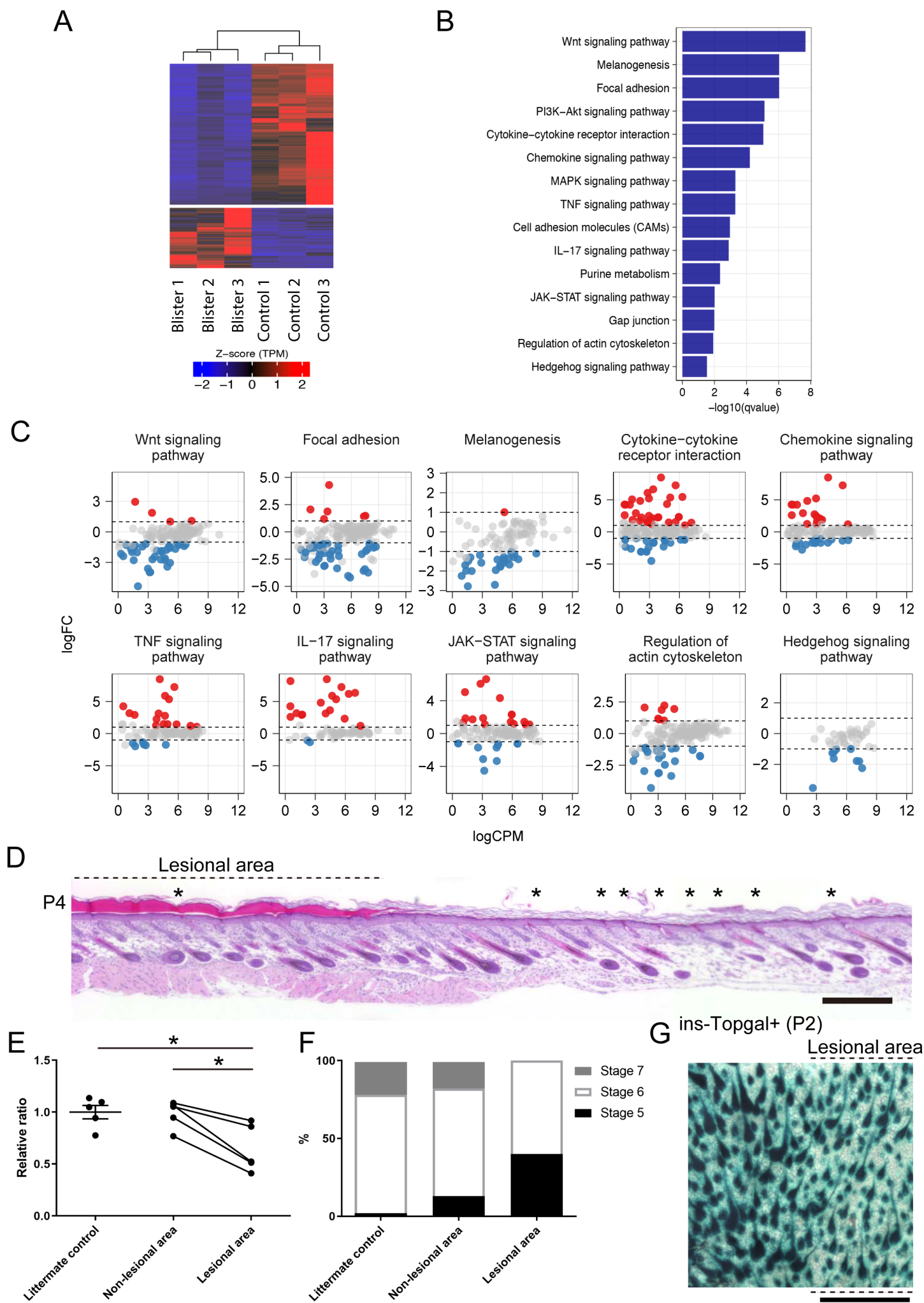


Figure 3. Predominant contribution of HF-derived keratinocytes to subepidermal blister healing

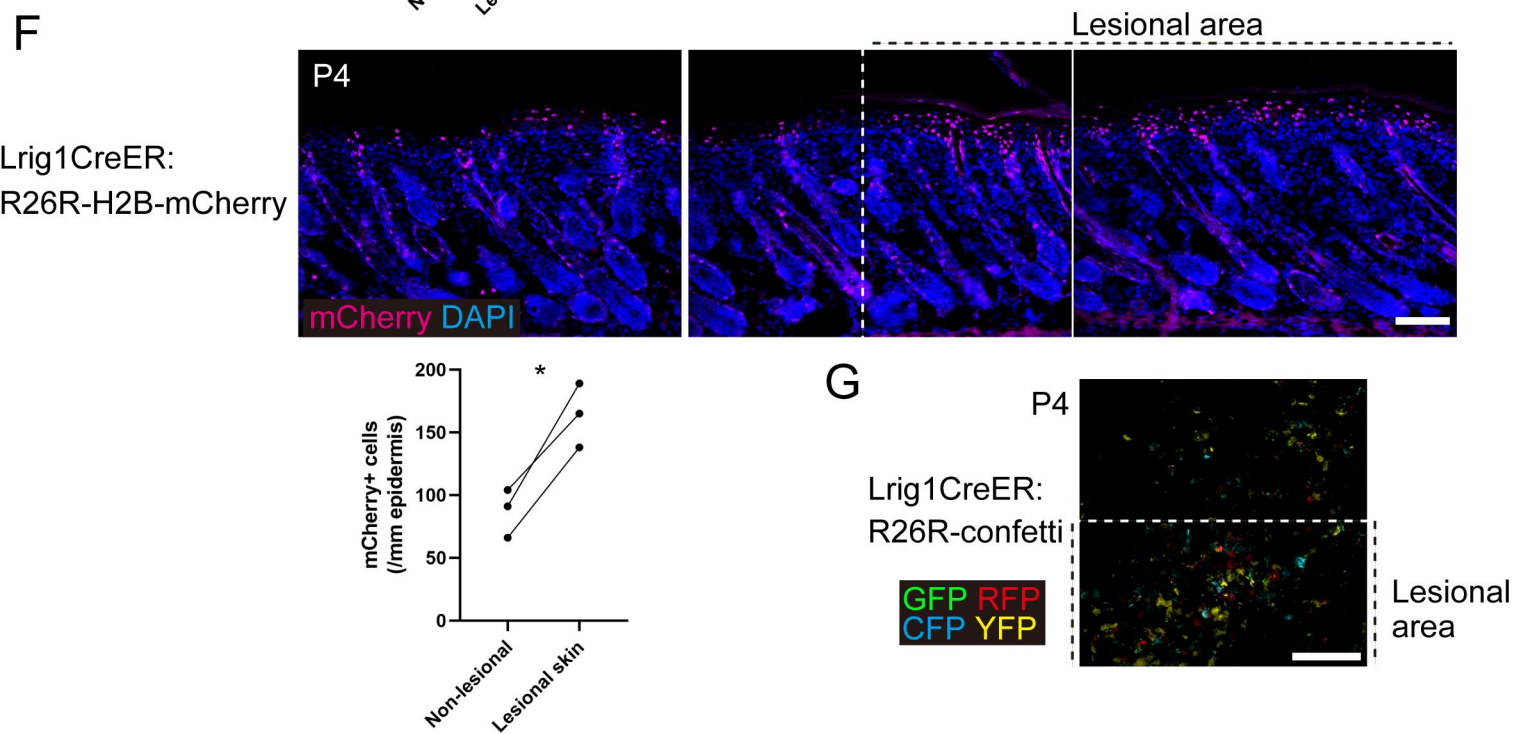
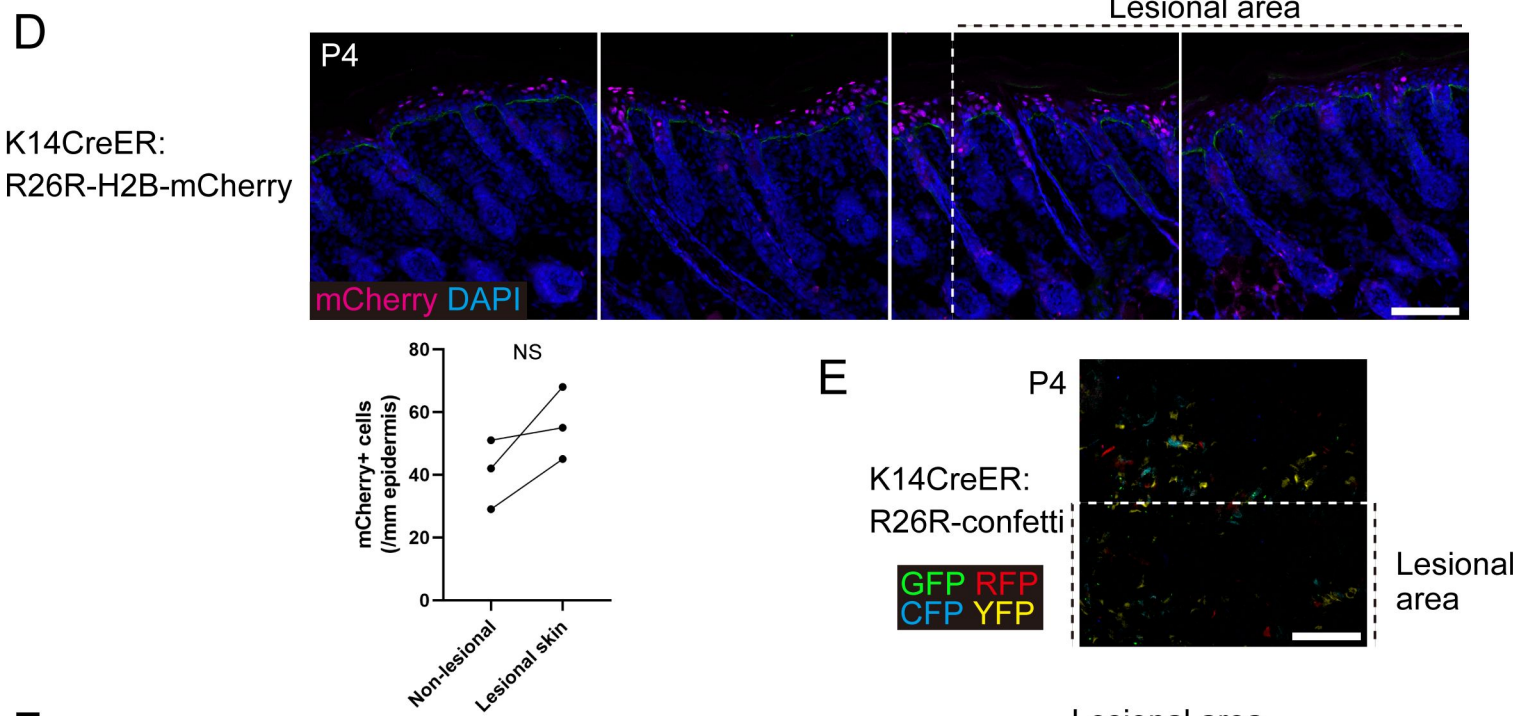
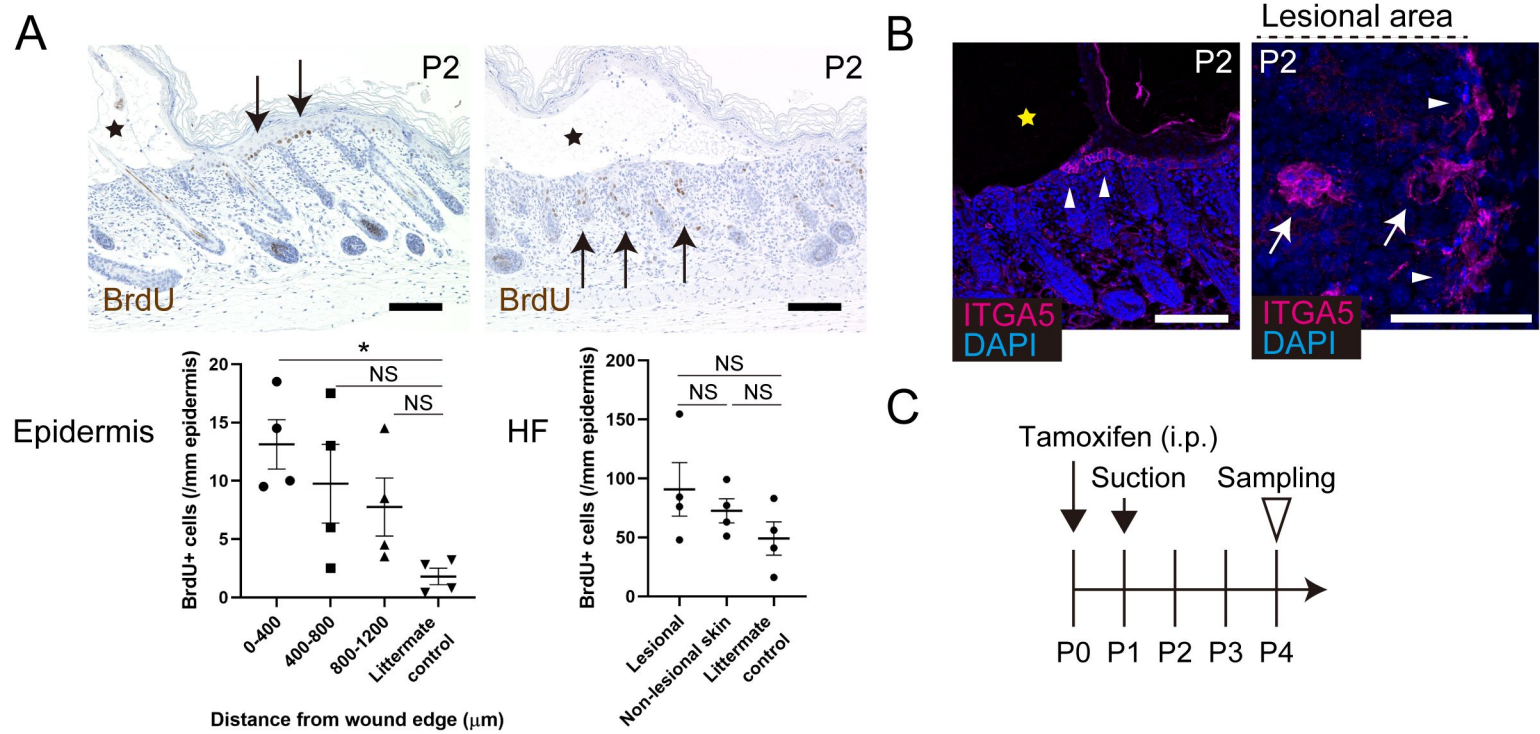


Figure 4. Effects of HF reduction on subepidermal blister healing

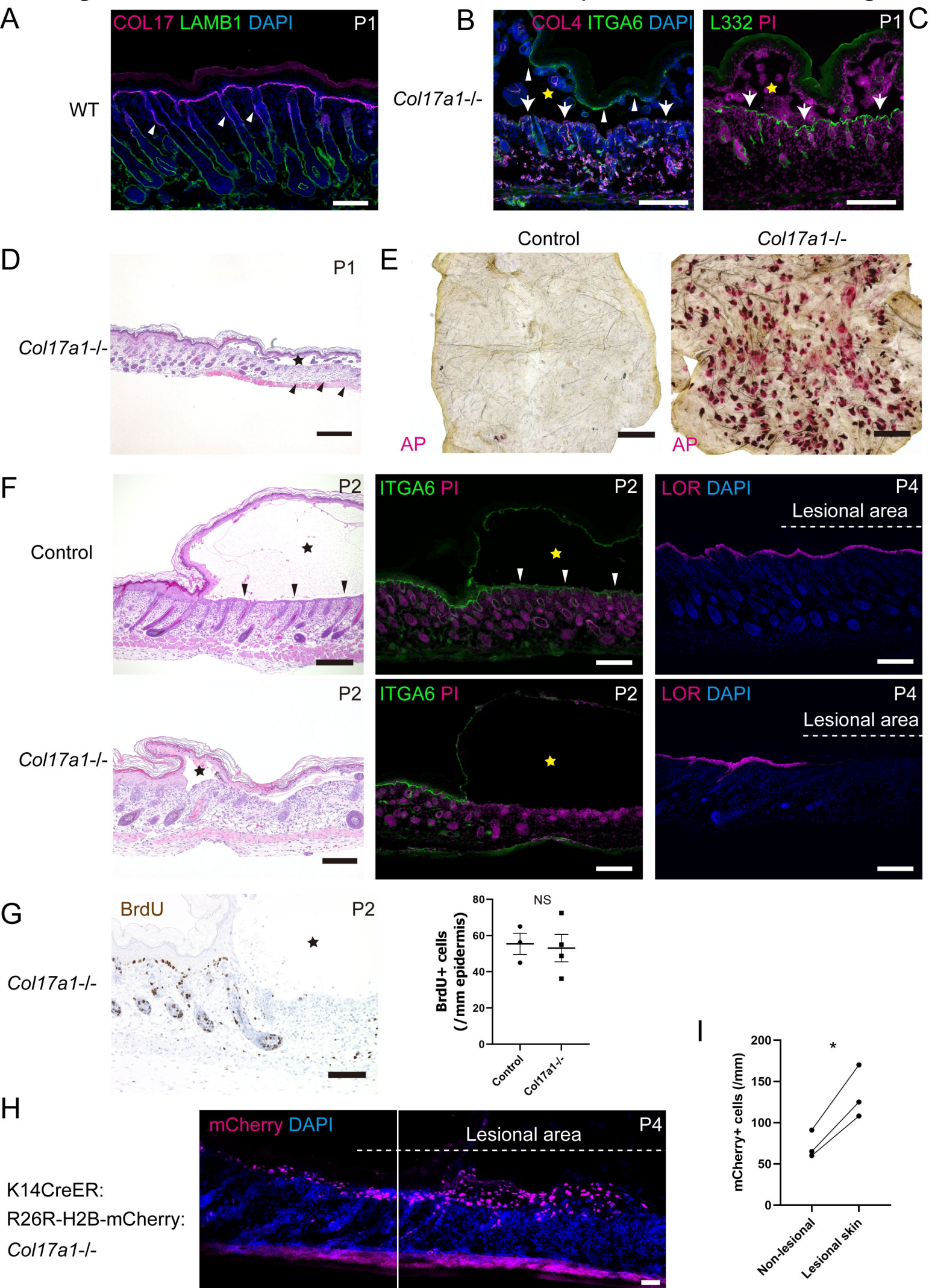


Figure 5. Involvement of keratinocyte shape transformation in subepidermal blister healing

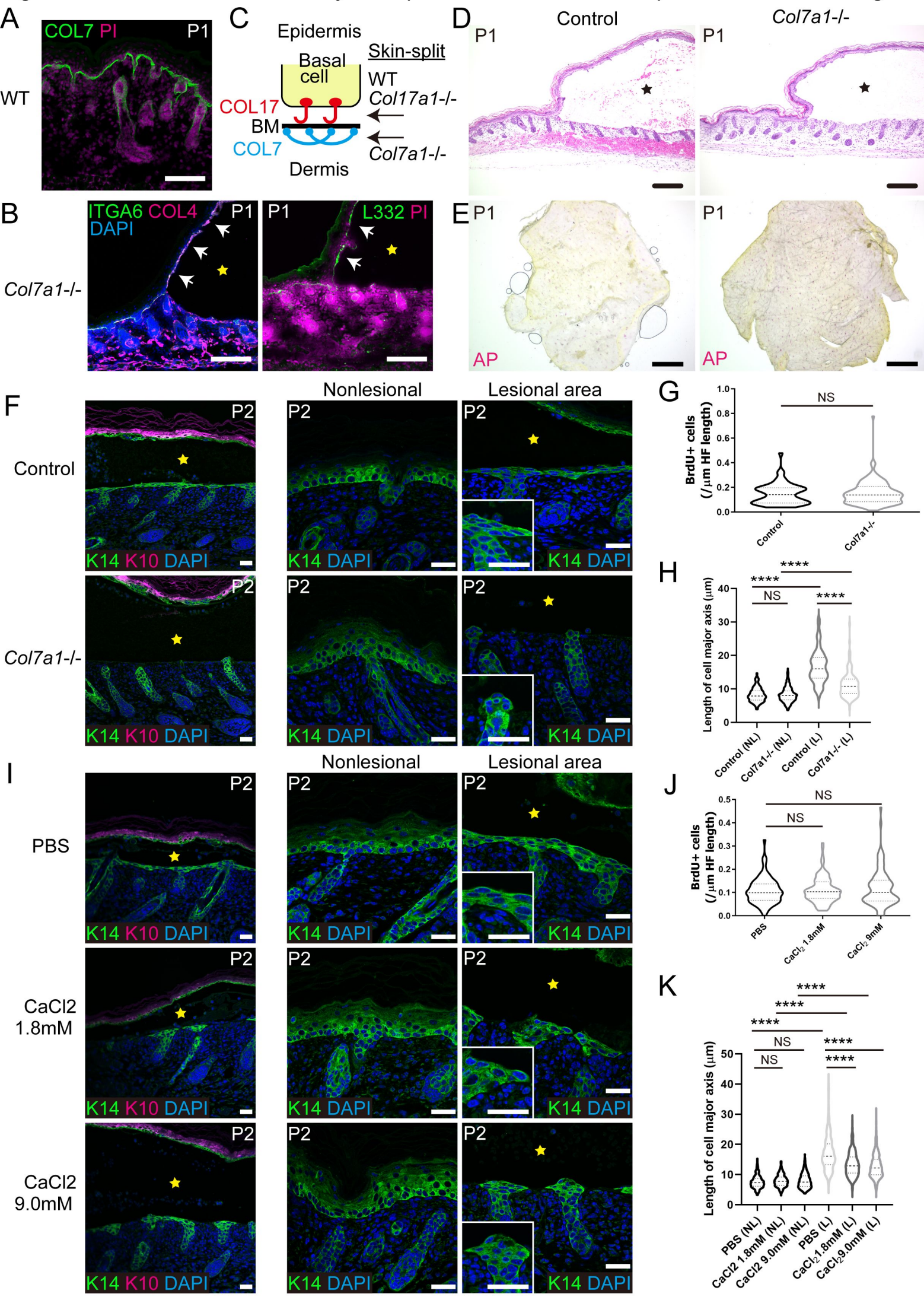
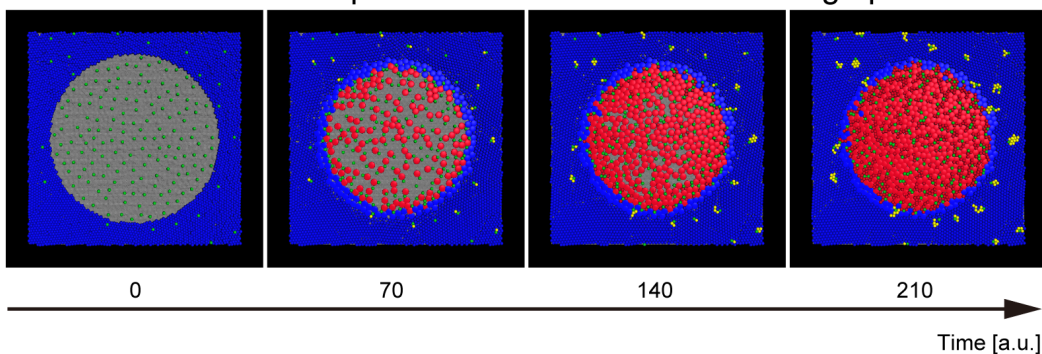


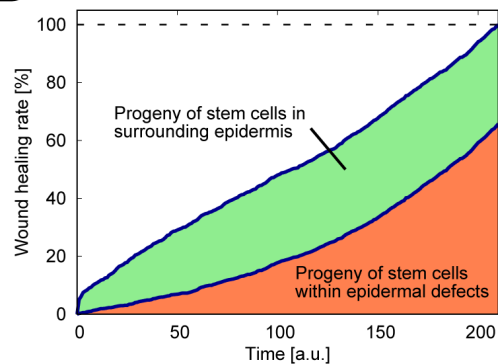
Figure 6. Mathematical modeling of subepidermal blister healing

A

Stem cells: both in epidermal defects and surrounding epidermis

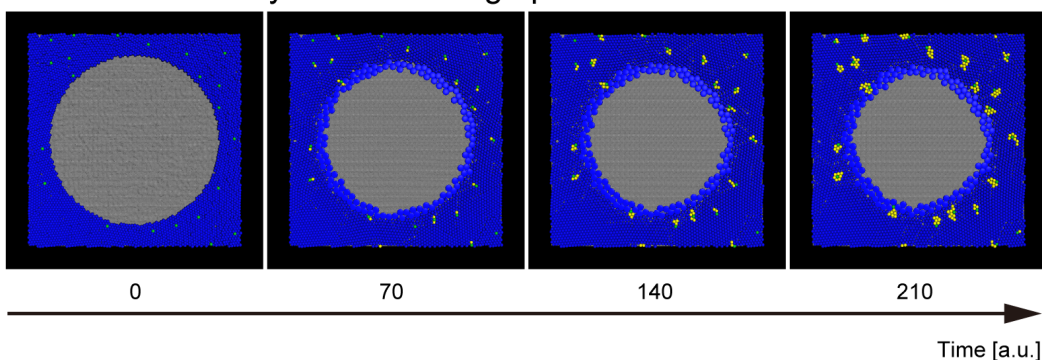


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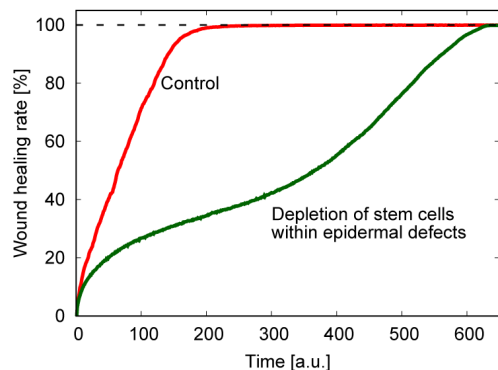


C

Stem cells: only in surrounding epidermis

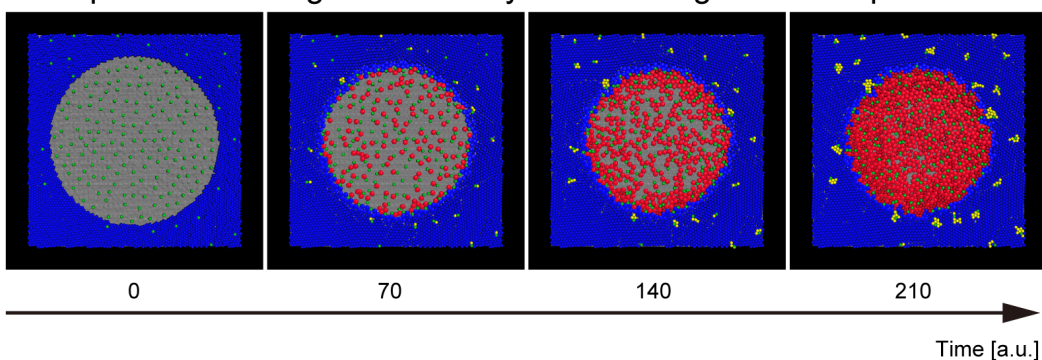


D



E

Impaired flattening of keratinocytes in the regenerated epidermis



F

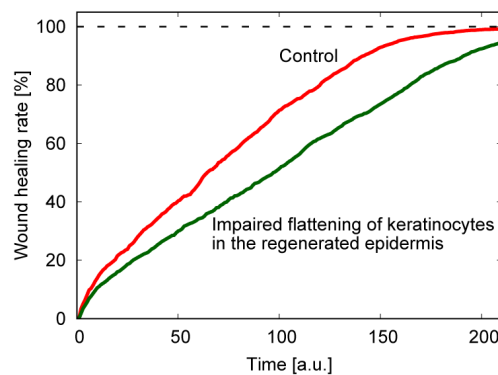


Figure EV1. Healing processes of subepidermal blisters

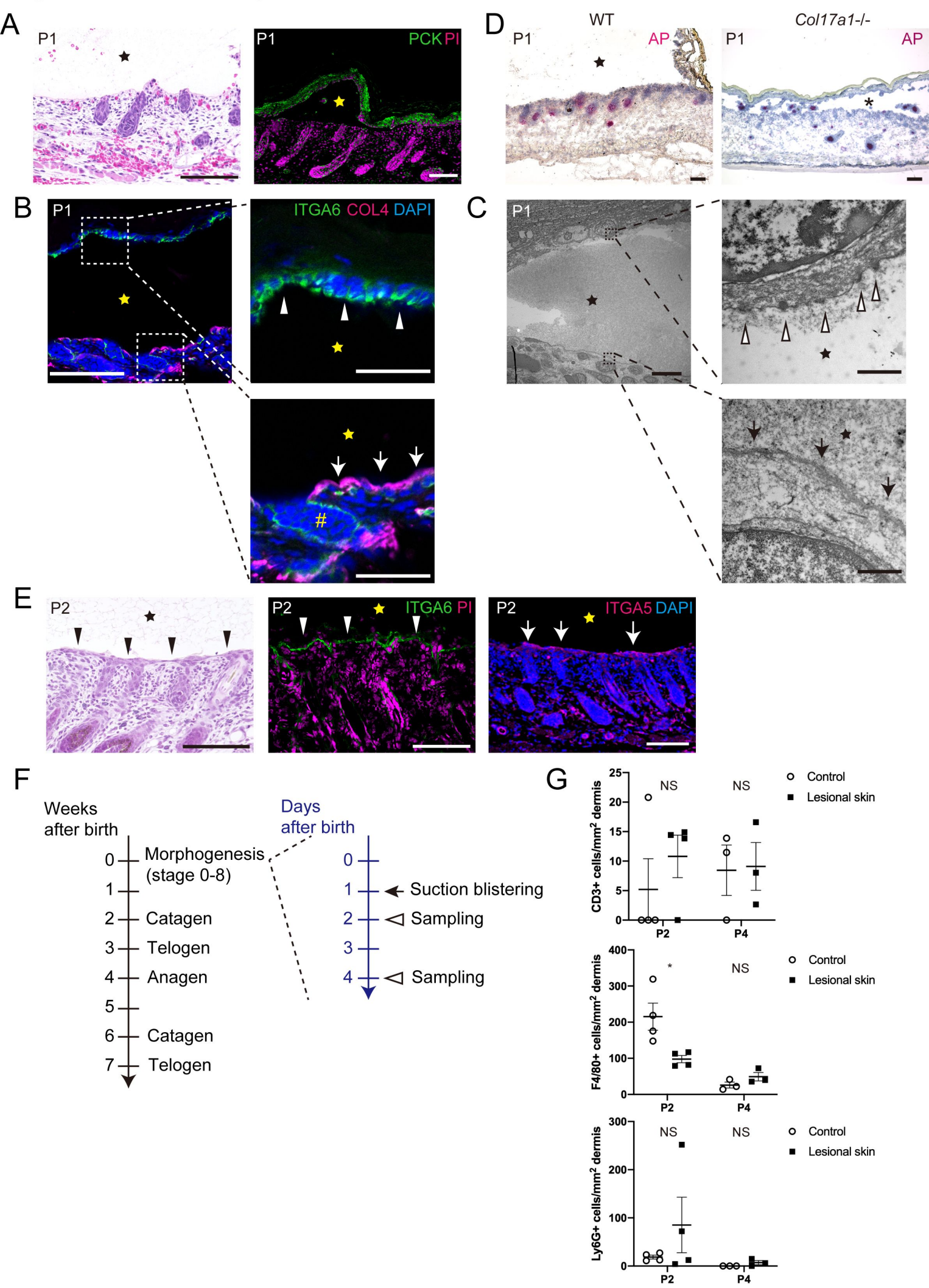
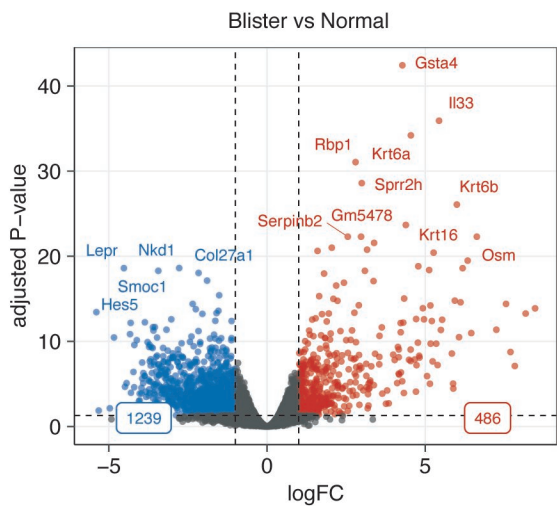
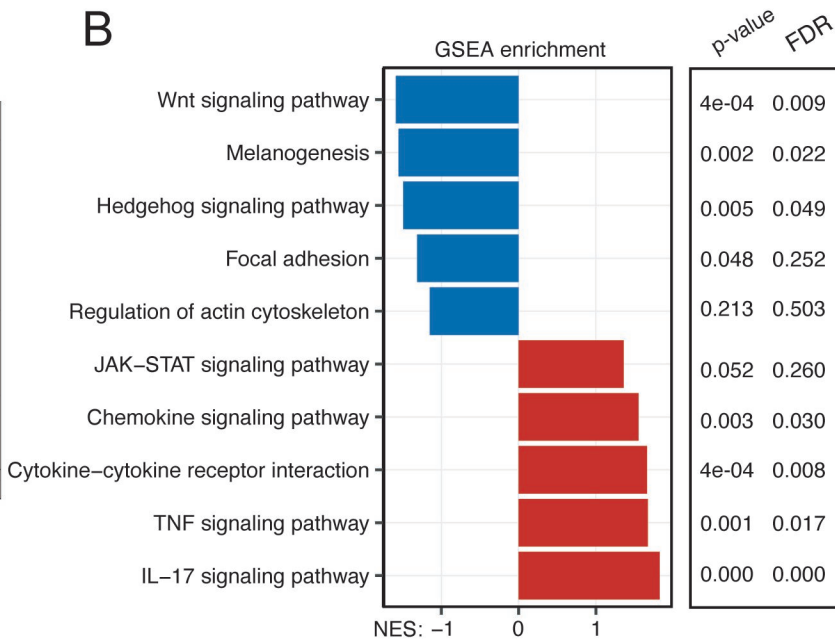


Figure EV2. RNA-seq on blister healing

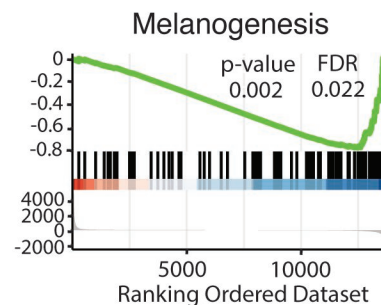
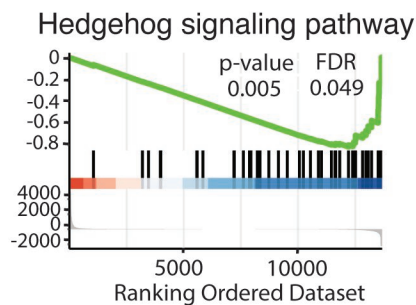
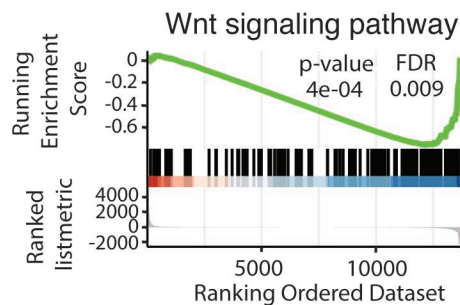
A



B



C



D

Down-regulated (blue square) Up-regulated (red square)

GO: Biological Process

GO: Molecular Function

GO: Cellular Component

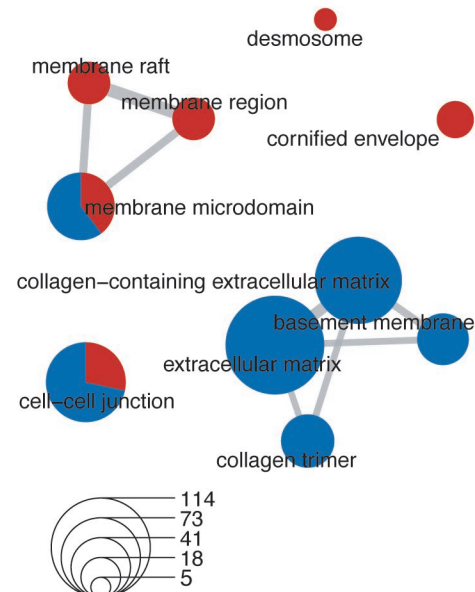
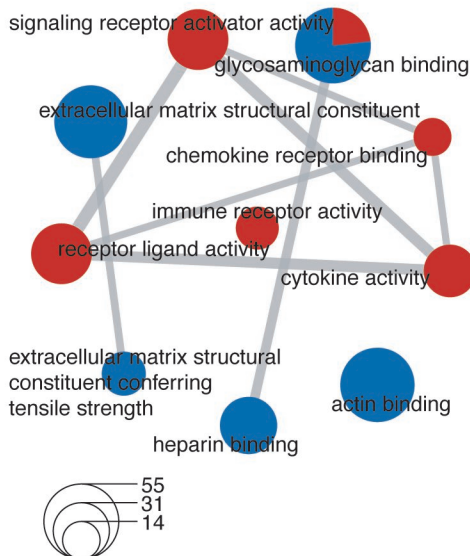
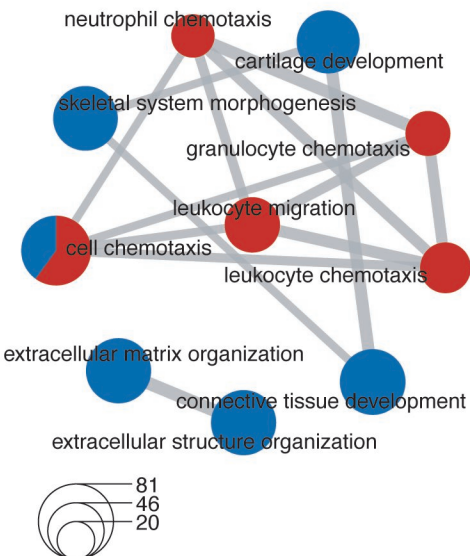


Figure EV3. Lineage tracing of subepidermal blister healing

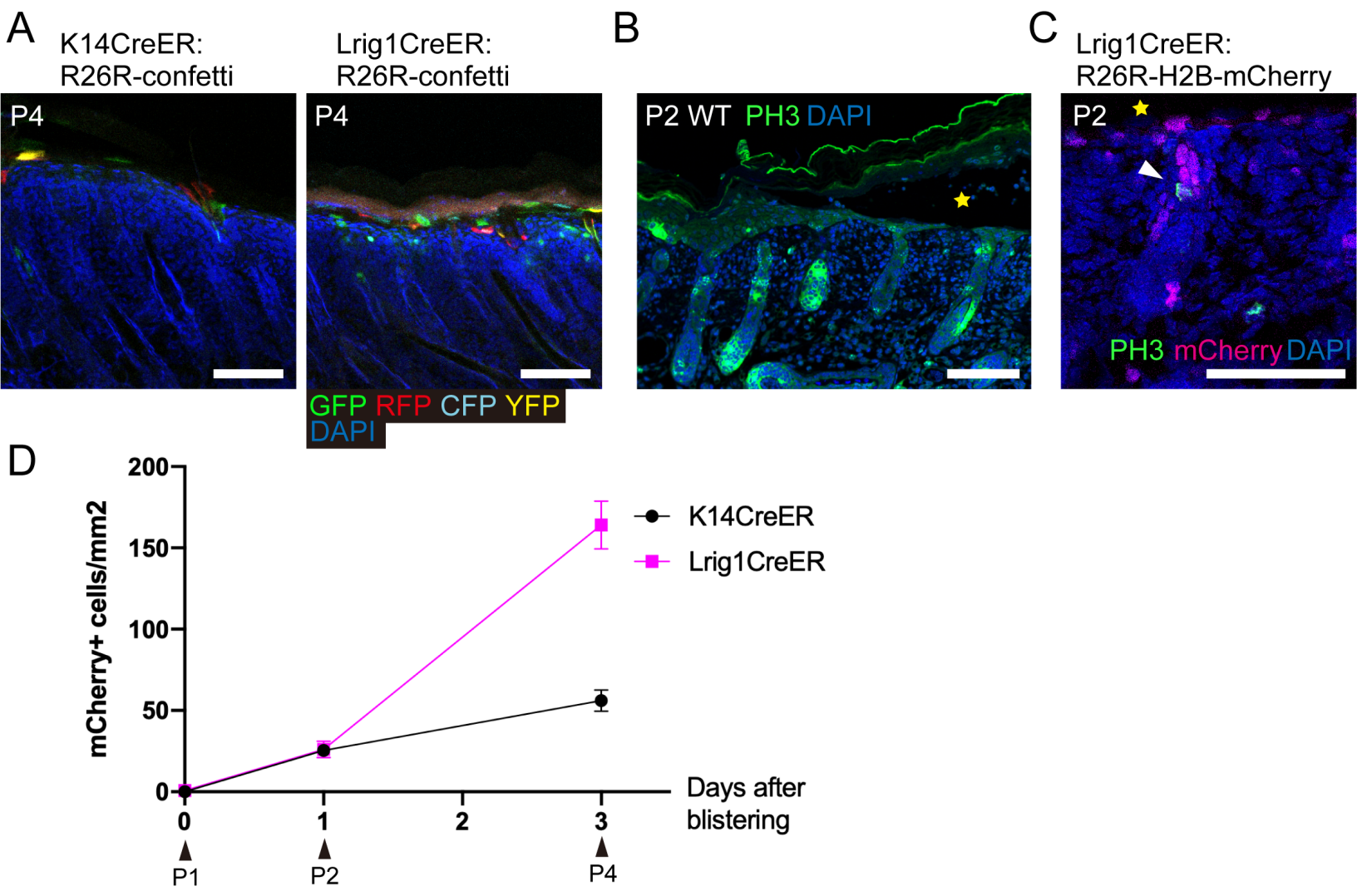


Figure EV4. Dependence of subepidermal blister healing on flattening ratio and SC arrangement

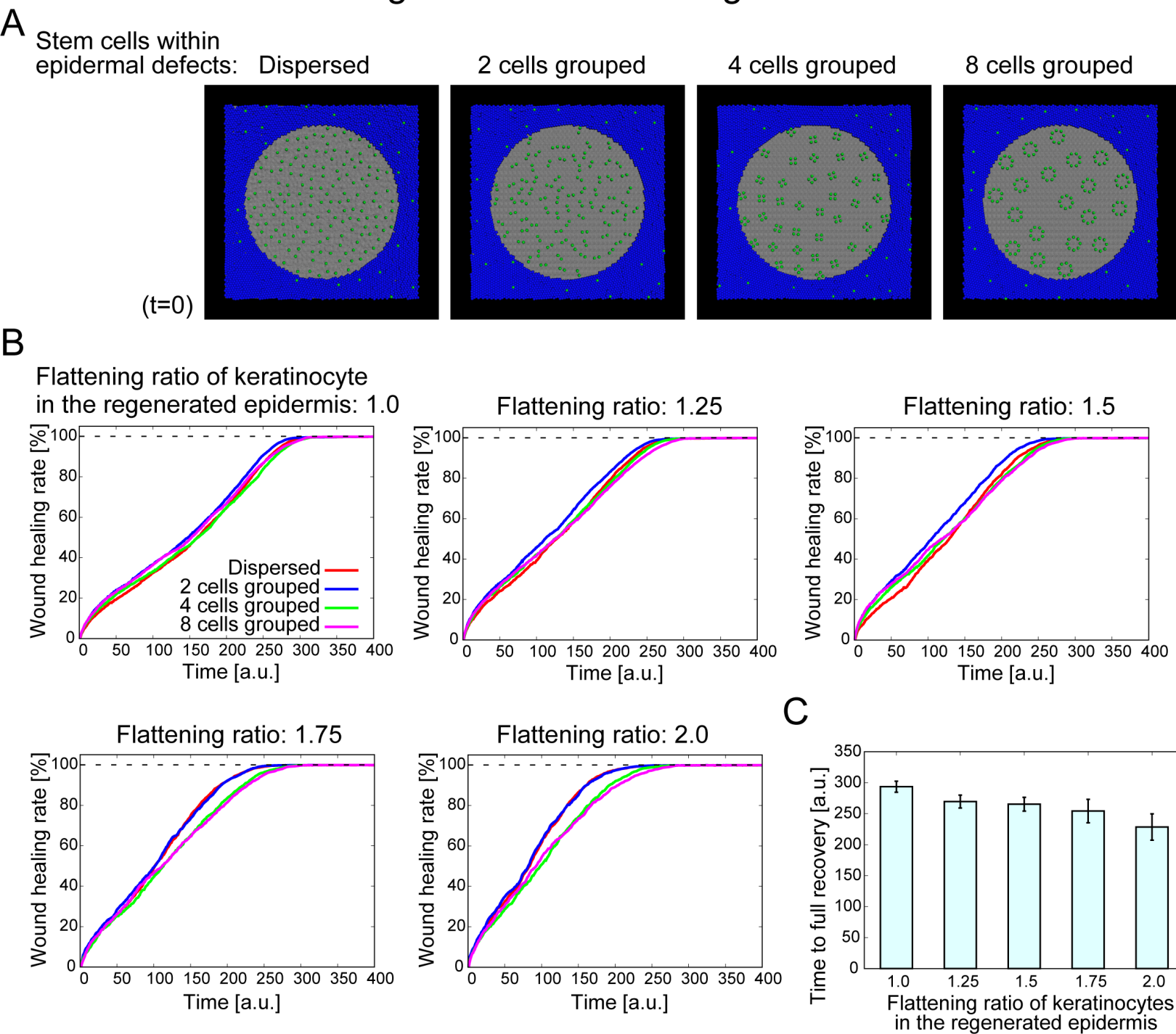
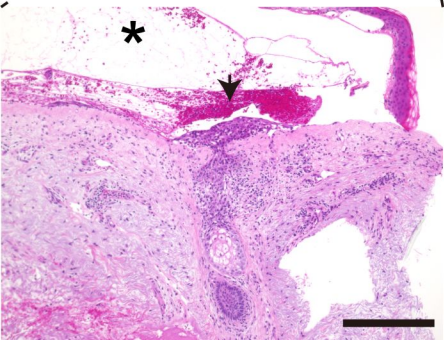
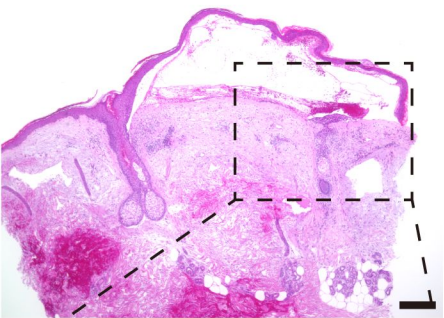
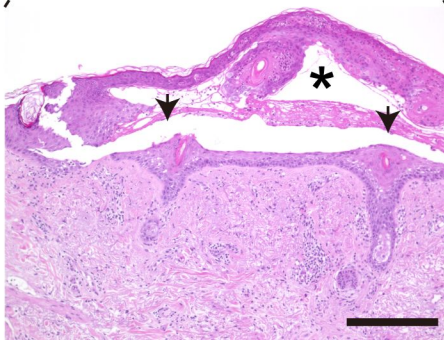
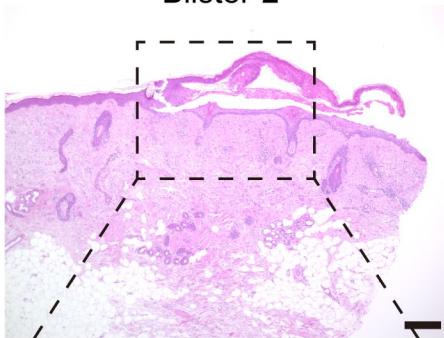


Figure EV5. Subepidermal blister healing in humans

Blister-1



Blister-2



Blister-3

