

ILK modulates epithelial polarity and matrix formation in hair follicles

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ABSTRACT Hair follicle morphogenesis requires coordination of multiple signals and communication between its epithelial and mesenchymal constituents. Cell adhesion protein platforms, which include integrins and integrin-linked kinase (ILK), are critical for hair follicle formation. However, their precise contribution to this process is poorly understood. We show that in the absence of ILK, the hair follicle matrix lineage fails to develop, likely due to abnormalities in development of apical–basal cell polarity, as well as in laminin-511 and basement membrane assembly at the tip of the hair bud. These defects also result in impaired specification of hair matrix and absence of precortex and inner sheath root cell lineages. The molecular pathways affected in ILK-deficient follicles are similar to those in the absence of epidermal integrin $\beta 1$ and include Wnt, but not sonic hedgehog, signaling. ILK-deficient hair buds also show abnormalities in the dermal papilla. Addition of exogenous laminin-511 restores morphological and molecular markers associated with hair matrix formation, indicating that ILK regulates hair bud cell polarity and functions upstream from laminin-511 assembly to regulate the developmental progression of hair follicles beyond the germ stage.

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INTRODUCTION

The formation of skin appendages involves an intricate series of signaling cascades and reciprocal modulation between neighboring epithelial and mesenchymal cells (reviewed in Millar, 2002; Fuchs, 2007; Duverger and Morasso, 2009). In mice, hair follicles begin to develop during organogenesis, when clusters of specialized mesenchymal cells derived from the dermomyotome, which later form the dermal papilla, begin to produce fibroblast growth factors and bone morphogenetic protein inhibitors. These messages stimulate the adjacent ectoderm, in which Wnt activity is induced to promote epithelial thickening and lymphoid enhancer factor 1 (LEF-1)–dependent formation of placodes. Placode development requires sustained Wnt-dependent processes, as well as activation of sonic hedgehog and other signaling pathways. After the first signals, a second wave of mesenchymal messages generated from the dermal papilla occurs

and is transmitted to adjacent epithelial cells in the hair placodes. These signals maintain the epithelial cells at the tip of the follicle in a proliferative state, inducing them to adopt an arrangement around the dermal papilla and allowing further follicle downgrowth.

The epithelial cells that surround the dermal papilla form the hair follicle matrix. These proliferative cells respond to differentiation cues, which induce them to withdraw from the cell cycle and move outward. Those matrix cells located at the center of the follicle differentiate to give rise to precortical cells, which later mature to generate the central medulla, the thick cortex, and an outer cuticle that form the hair shaft. A different subpopulation of matrix cells that surround those at the center serve as progenitors for the inner root sheath upon differentiation.

Cell adhesion molecules and proteins involved in attachment to extracellular matrix substrates play critical but poorly understood roles in hair follicle development and maintenance (El-Amraoui and Petit, 2010). For example, analysis of perinatal mice with epidermis-restricted inactivation of the genes encoding integrin $\beta 1$ or integrin-linked kinase (ILK) shows abnormal hair follicles or hair follicle degeneration after birth, depending on the strain analyzed (Brakebusch *et al.*, 2000; Raghavan *et al.*, 2000; Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008). Significantly, the molecular mechanisms involved in these abnormalities remain unexplored.

Integrin-linked kinase is a ubiquitous scaffold protein that interacts with multiple factors and modulates a variety of cellular

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Abbreviations used: aPKC, atypical protein kinase C; GSK-3 β , glycogen synthase kinase 3 β ; ILK, integrin-linked kinase; PBS, phosphate-buffered saline.

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responses. Subsets of ILK-binding proteins are associated with integrin and/or growth factor receptor stimulation (Ho *et al.*, 2009; Ho and Dagnino, 2012a,b). In the epidermis, ILK fulfills pivotal physiological roles, as evidenced by the broad transcriptome alterations and pleiotropic abnormalities observed in mice with epidermis-restricted inactivation of *Ilk* (Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008; Judah *et al.*, 2012). More specifically, ILK is a major modulator of actin cytoskeletal and microtubule dynamics, development of front–rear polarity, and directional migration in keratinocytes (Nakrieko *et al.*, 2008; Ho *et al.*, 2009; Ho and Dagnino, 2012a; Wickstrom *et al.*, 2010a,b). It also mediates normal activation of Rac1 in response to stimulation by epidermal or keratinocyte growth factors, and, in so doing, it modulates the capacity of keratinocytes to phagocytose melanosomes, thus regulating cutaneous pigmentation (Ho and Dagnino, 2012b; Sayedyahosseini *et al.*, 2012). In the postnatal hair follicle, expression of ILK in bulge stem cells is required for generation of stem cell progeny that must migrate away from this niche to repopulate the epidermis during skin regeneration after injury (Nakrieko *et al.*, 2011). Of note, although it is clear that inactivation of the *Ilk* gene in the embryonic epidermis results in impaired hair follicle formation, virtually nothing is understood about the precise molecular functions that ILK modulates during hair follicle development. We now address this critical issue, showing that ILK-deficient hair follicles arrest before stage 3/4 of development, and provide evidence of impaired development of keratinocyte polarity and generation of matrix cells, necessary for the subsequent formation of multiple hair follicle lineages. Our studies are the first to elucidate the molecular alterations in hair follicle morphogenesis caused by disruption of integrin-associated signaling platforms.

RESULTS

Developmental arrest of hair follicle morphogenesis in ILK-deficient epidermis

Mice with epidermis-restricted inactivation of the *Ilk* gene, hereafter termed *K14Cre;Ilk^{fl/fl}*, die perinatally. Analysis of these mice at 1–4 d of age showed impaired epidermal integrity and severe defects in hair follicles (Nakrieko *et al.*, 2008). Given that hair follicle formation initiates during organogenesis, and to investigate the mechanisms involved in ILK regulation of hair follicle morphogenesis, we first determined which stage in the development of this appendage is altered in the absence of ILK expression. The initial signals that direct hair follicle formation in mice arise from the mesenchyme around embryonic day (E) 14.5 and result in thickening of the overlying ectodermal epithelium to form a placode (Supplemental Figure S1; Millar, 2002; Duverger and Morasso, 2009). Placode cells proliferate and signal to the underlying mesenchyme, inducing condensation of dermal cells to form the dermal papilla, thus giving rise to the hair germ. The epithelial cells of the hair germ continue to proliferate and invaginate into the dermis, generating the hair peg around E17.5. At this stage, the follicular cells progressively adopt an arrangement to surround the dermal papilla, giving rise to the bulbous peg (Supplemental Figure S1). Bidirectional signaling between the epithelium and the mesenchymal cells of the dermal papilla is established and is essential to drive further growth of the follicle and differentiation of follicular keratinocytes.

Examination of ILK-deficient epidermis from E15.5 *K14Cre; Ilk^{fl/fl}* mice indicated that formation of ectodermal cell condensates proceeds without detectable alterations relative to ILK-expressing tissues (Figure 1A and Supplemental Figure S2). In E16.5 animals, we found a slight decrease in the abundance of follicular structures. However, substantial abnormalities became evident in E17.5 mice.

Specifically, ILK-deficient hair follicles failed to reach the bulbous peg stage, and the epithelial cells did not develop to surround the dermal papilla, a defect that persisted in 2-d-old animals (Figure 1A and Supplemental Figure S2).

To determine whether proliferation defects were associated with the markedly abnormal development of ILK-deficient follicles, we assessed Ki67 expression. We found a small but statistically significant decrease in the fraction of Ki67-positive cells in E17.5 ILK-deficient follicle cells, whereas the proportion of Ki67-positive cells in interfollicular keratinocytes was not detectably altered by *Ilk* gene inactivation (Figure 1B). We also examined whether changes in cell viability could account for the observed phenotype. Of note, we did not detect apoptotic cells in E17.5 follicles, irrespective of the presence or absence of ILK (Figure 1C). In contrast, a substantial proportion of apoptotic ILK-deficient follicular keratinocytes was evident in 3-d-old mice (Figure 1C). To examine the possibility that the development of ILK-deficient hair follicles may be simply delayed, we harvested skins from 2-d-old *K14Cre; Ilk^{fl/fl}* mice or ILK-expressing *K14Cre; Ilk^{fl/+}* littermates and grafted them onto *Cr1:NU-Foxn1^{nu}* immunodeficient mice. Whereas ILK-expressing grafts produced abundant hair, no hair or hair follicles were detected in ILK-deficient grafts as late as 10 wk after transplantation (Figure 1D), suggesting that the wave of apoptosis present postnatally may result in hair follicle regression. We conclude that ILK expression is necessary for hair follicles to advance to stage 3/4.

Abnormal activation of developmental signaling pathways in ILK-deficient hair follicles

Pelage formation in the mouse involves several waves of hair follicle induction, and the canonical Wnt/ β -catenin pathway is central in this process. Activation by Wnt occurs during placode formation and is involved in subsequent stages of hair follicle morphogenesis (Supplemental Figure S1). This signaling pathway also involves expression and nuclear localization of LEF-1, which mediates the transcriptional activation of a variety of Wnt target genes (Millar, 2002; Duverger and Morasso, 2009). Analysis of LEF-1 expression in developing follicles of E15.5 or E16.5 embryos did not reveal any major differences between ILK-expressing and ILK-deficient epidermis (Supplemental Figure S2A). In normal E17.5 hair pegs, nuclear LEF-1 immunoreactivity was readily observed in epithelial cells directly adjacent to dermal papilla, as well as in dermal papilla fibroblasts (Figure 2A). In contrast, LEF-1 immunoreactivity in E17.5 ILK-deficient follicles appeared weaker and was not present in all cells adjacent to the dermal papilla (Figure 2A). To further investigate this issue, we determined by quantitative PCR (qPCR) the abundance of various transcripts associated with Wnt signaling in E17.5 dorsal skin. We observed significant reduction in the levels of *Cdh1*, *Cdh3*, and *Wnt3A* transcripts, which encode, respectively, E-cadherin, P-cadherin, and Wnt3A. The last is a key hair-inducing factor for dermal papilla cells (Kishimoto *et al.*, 1999). We also confirmed the reduced abundance of P-cadherin protein in dorsal skin lysates of these animals (Supplemental Figure S3, B and C). These observations suggest that abnormalities in Wnt signaling are associated with ILK deficiency around the time follicles approach the hair peg stage.

Sonic hedgehog (Shh) is a morphogen secreted in the follicular placode, mediates key events necessary for epithelium–dermal papilla communication, and is essential for the formation of mature hair follicles (St. Jacques *et al.*, 1998; Millar, 2002; Woo *et al.*, 2012). Although Shh signaling is not necessary for placode initiation, it is required for hair follicle development beyond stage 2. Shh is also involved in maintenance of dermal papilla identity and its hair-inducing

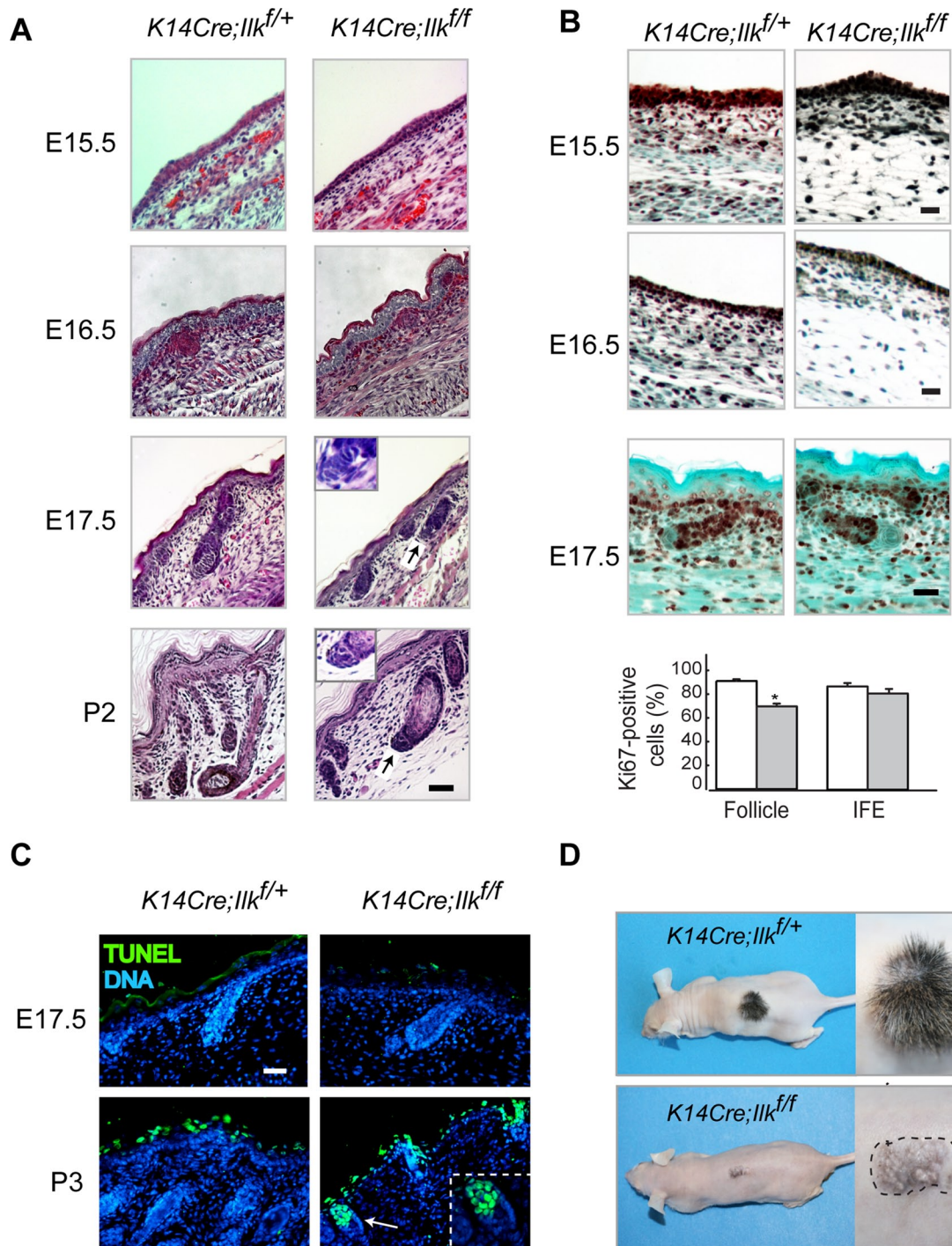


FIGURE 1: Developmental arrest of ILK-deficient hair follicles. (A) Epidermal sections from mice with the indicated genotypes and ages were stained with hematoxylin and eosin. Mesenchymal condensates corresponding to the dermal papilla indicated by arrows are shown in the insets at higher magnification. (B) Epidermal sections from the animals described in A were processed for immunohistochemistry, using anti-Ki67 antibodies. The histograms represent the fraction of Ki67-positive cells present in the hair pegs or the interfollicular epidermis (IFE) from ILK-expressing (white bars) or ILK-deficient (gray bars) mice and are expressed as average + SEM ($n = 6$). * $p < 0.05$ (Student's t test). (C) Epidermal sections from the animals described in A were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling to detect apoptotic cells. Nuclear DNA was visualized with Hoechst 33342. The P3 follicle indicated with the arrow is shown at higher magnification in the inset. (D) Skin isolated from 2-d-old mice with the indicated genotype was grafted onto immunodeficient *CrI:NU-Foxn1^{nu}* mice and is shown 10 wk posttransplantation. The dashed line indicates the edge of the ILK-deficient skin graft, identifiable by its pigmentation. Bar, 32 μm (all micrographs).

properties (Yang and Cotsarelis, 2010). Within the follicular epithelium, Shh stimulates proliferation, acting coordinately with other factors, such as Noggin, which functions to inhibit BMP signaling and

promotes hair follicle downgrowth. Given the phenotypic similarities between ILK- and Shh-deficient hair follicles, we next examined the expression of *Gli1* and *Wnt5a* in E17.5 *K14Cre;Ilk^{f/f}* embryos, as both

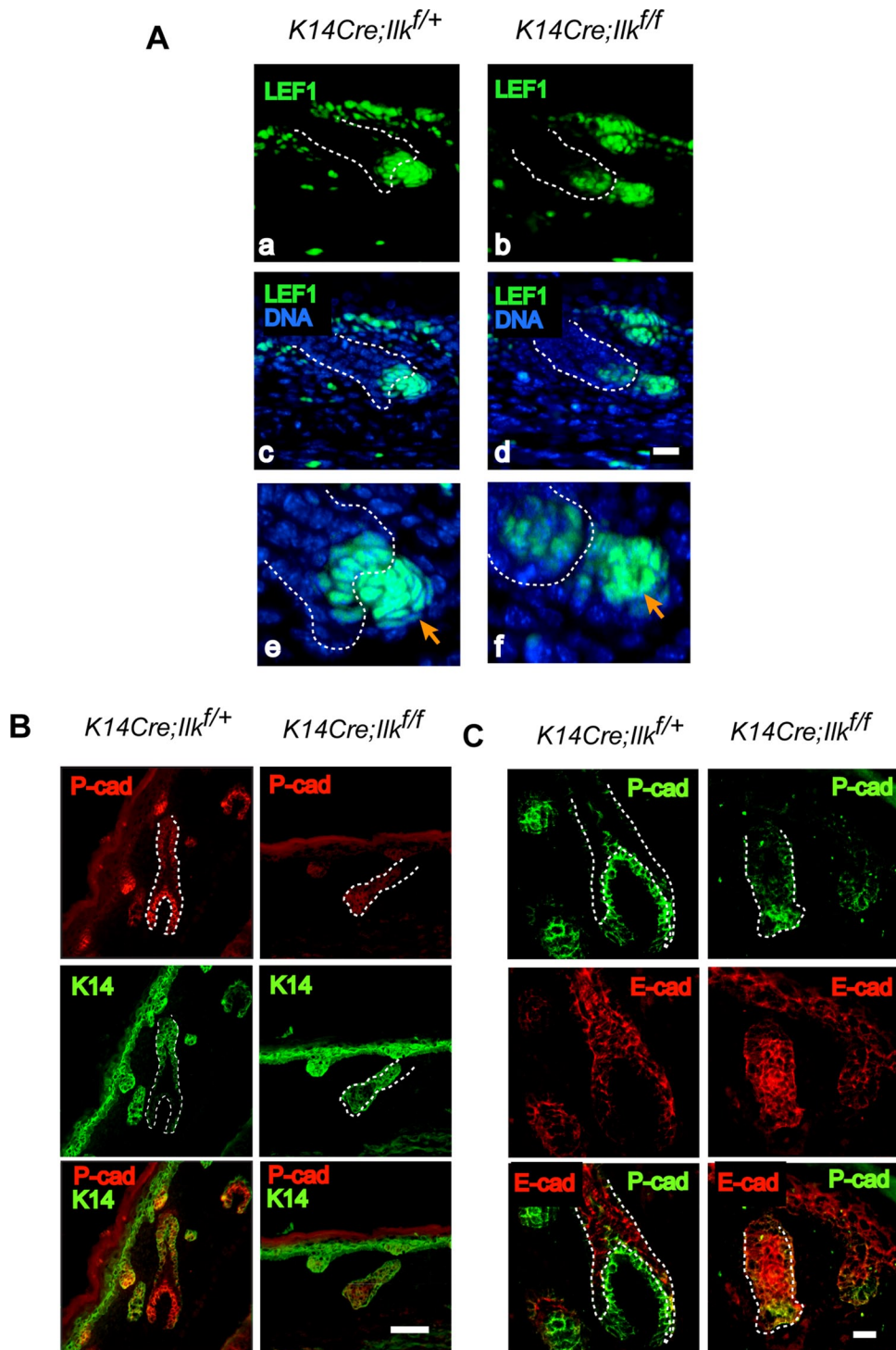


FIGURE 2: Abnormal Wnt signaling and matrix development in ILK-deficient hair follicles. Dorsal tissue sections from E17.5 (A, B) or E19.5 (C) embryos with the indicated genotypes were processed for immunofluorescence microscopy, using antibodies against LEF-1, P-cadherin (P-cad), keratin 14 (K14), or E-cadherin (E-cad). Nuclear DNA was visualized with Hoechst 33342. The arrows in A indicate the dermal papilla, and dotted lines indicate the borders of the hair follicles, except in the micrographs showing *K14Cre;Ilk^{f/+}* tissues in C, in which they indicate E-cadherin-positive and P-cadherin-excluding areas. Bar, 32 μ m.

are direct Shh targets, and *Gli1* also mediates many effects of this factor. Using in situ hybridization and qPCR, we found no detectable abnormalities in the expression of *Gli1* and *Wnt5a* mRNA in ILK-deficient follicles. Further, the levels of mRNAs encoding Noggin, BMP receptor type 1A, and BMP6 were also normal in ILK-deficient skin

(Supplemental Figure S3, B and D). On the basis of these observations, we propose that ILK is not essential for at least a subset of immediate responses to Shh-mediated stimulation, such as *Gli1* transcription. ILK does not appear to be required for normal transcription of other Shh target genes, such as *Noggin* and *Bmpr1A*.

ILK is necessary for formation of the hair follicle matrix

P-cadherin is expressed in hair follicle matrix keratinocytes. This protein is reportedly involved in fate decisions that result in the specification of matrix cells into a distinct subpopulation regulated by signals from adjacent dermal papilla fibroblasts (Muller-Rover *et al.*, 1999). The abnormalities in morphology and Wnt signaling observed in ILK-deficient hair follicles prompted us to examine the localization patterns of E- and P-cadherin. We observed that P-cadherin immunoreactivity in control, stage 4/5 *K14Cre;Ilk^{f/f}* hair pegs was restricted to inner matrix cells contiguous to the dermal papilla (Figure 2, B and C), as previously reported (Muller-Rover *et al.*, 1999). P-Cadherin immunoreactivity was not observed in all the cells forming ILK-deficient *K14Cre;Ilk^{f/f}* follicles. Further, and in contrast with normal hair pegs, P-cadherin was detectable in ILK-deficient keratinocytes located several cell layers away from the underlying condensed mesenchymal cells constituting the presumptive dermal papilla in E17.5 embryos (Figure 2, B and C).

E-cadherin is another important junctional protein in the hair follicle (Paus *et al.*, 1999). In ILK-expressing tissues, we observed E-cadherin immunoreactivity from stage 3 follicles onward. Of note, in ILK-expressing hair follicles that had developed to or past stage 4, E-cadherin was detected in all cells, with the exception of the P-cadherin-positive inner matrix cells (Figure 2C). The presence of mutually exclusive regions of E- and P-cadherin expression in hair pegs suggests the acquisition of distinct identities in various cell subpopulations, possibly in response to specific signals that arise as the hair follicle matures and forms matrix cell precursors and descendants. In contrast, E-cadherin was present in all ILK-deficient cells, irrespective of whether they also expressed P-cadherin. These overlapping E- and P-cadherin expression patterns are normally limited to stages 1–3 of hair follicle development (Muller-Rover *et al.*, 1999) and suggest that ILK deficiency is linked to developmental arrest, with alterations in the mechanisms that specify matrix keratinocytes.

Inner root sheath cell lineage deficiency in the absence of ILK

The cells in the hair follicle matrix are undifferentiated progenitors that maintain their properties through interactions with cells in the dermal papilla (Cotsarelis *et al.*, 1990). Matrix cells can generate an intermediate population of transiently dividing progenitor cells. The latter differentiate to give rise to the hair shaft and the inner root sheath lineages through processes that involve, respectively, Wnt signaling and activation of GATA-3 expression and its transcriptional gene targets (Kaufman *et al.*, 2003).

To further explore the abnormalities in ILK-deficient hair follicles, we next examined the expression and phosphorylation status of glycogen synthase kinase 3 β (GSK3- β). This kinase can be regulated by multiple, independent mechanisms and is involved in distinct pathways, including those implicated in canonical Wnt signaling. GSK3- β is inactivated by phosphorylation on Ser-9, which can result in the initiation of various signaling cascades, some of which lead to transcriptional up-regulation of LEF-1/ β -catenin targets (Wu and Pan, 2010). We observed the presence of GSK3- β throughout the hair follicle, irrespective of the presence or absence of ILK (Figure 3A). In ILK-expressing follicles, we also observed phospho-GSK3- β immunoreactivity in a subset of cells in the hair matrix, as well as in regions likely corresponding to the inner root sheath (Figure 3A). Significantly, we did not detect any phosphorylated GSK3- β in ILK-deficient follicles, providing additional evidence to support the proposal that at least some aspects of Wnt signaling are defective in the absence of ILK and suggesting that ILK deficiency also leads to

impairment in the specification of hair matrix-derived cells. To address this latter possibility, we investigated the presence of GATA-3. This transcription factor is present in and necessary for inner root sheath cell specification. In agreement with previous reports (Kaufman *et al.*, 2003), we observed GATA-3 immunoreactivity in a narrow set of cells above the hair matrix and in a pattern that partially overlapped with areas that were positive for phospho-GSK3 β (Figure 3B). In stark contrast, we did not detect GATA-3 in ILK-deficient hair follicles (Figure 3B), indicating that *Ilk* inactivation is associated with absence of inner root sheath cell populations. In agreement with the notion that ILK-deficient hair follicles fail to generate matrix cells and their IRS descendants, our microarray analysis of epidermal tissues isolated from 3-d-old ILK-deficient mice (Judah *et al.*, 2012) revealed significant decreases in the expression of other genes involved in hair matrix formation and/or differentiation into inner root sheath cells. These genes include *Dlx3*, a Wnt-regulated transcription factor, which, in turns, modulates GATA-3 expression (Hwang *et al.*, 2008), and *Msx2*, which is present in the hair matrix and is essential for the differentiation of these cells into the inner root sheath (Cai *et al.*, 2009).

Loss of keratinocyte apical–basal polarity in the absence of ILK

The epidermis and the hair follicle are polarized, although in a manner somewhat different from simple vertebrate epithelia. Specifically, prototypical polarized epithelia, such as the intestinal epithelium, exhibit clear apical and basolateral membrane domains separated by apical junction complexes (Chen and Zhang, 2013). In contrast, the epidermis and the hair follicle consist of multiple layers of cells that establish polarity along the basal-to-apical axis of the tissue through poorly understood mechanisms. In particular, the developing hair follicle consists of several layers of epithelial cells that attach to each other via adherens and other junctions. The outermost cell layer also adheres to the surrounding basement membrane, and through this three-dimensional arrangement, cells develop polarity. One characteristic of apical–basal polarity is the asymmetric distribution of various proteins relative to the basal aspect of the cell, which is adjacent to the basement membrane.

Integrins and the actin cytoskeleton participate in the establishment of apical–basal polarity in simple epithelia (Yu *et al.*, 2005). Given that ILK is a key component of integrin activation pathways and also plays central roles in F-actin dynamics in keratinocytes (Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008), we hypothesized that alterations in cell polarity might precede and/or be associated with the observed abnormalities in ILK-deficient hair follicle development. Hence we investigated whether several key proteins in hair buds are distributed in a polarized manner, by analyzing single confocal optical sections of early-stage follicles at E16.5, before the observed impairment in the specification of hair matrix cells and inner root sheath precursors. At this early stage, many cells in both normal and ILK-deficient follicles coexpress E- and P-cadherin. Of note, in ILK-expressing follicles, these two adhesion proteins are readily observed on the lateral and “apical” aspects of the cells associated with the center of the follicle but not on the basal side of the cell, defined here as the region that contacts the basement membrane (Figure 4A). In stark contrast, in ILK-deficient follicles, E- and P-cadherin immunoreactivity was abundantly evident on plasma membrane regions adjacent to the basement membrane, as well as laterally (Figure 4A), indicating a substantial disruption of apical–basal polarity in the absence of ILK. To further examine this phenomenon, we also investigated the organization of F-actin and the distribution of integrin β 1 in E16.5 tissues. In ILK-expressing hair bud keratinocytes, thick actin

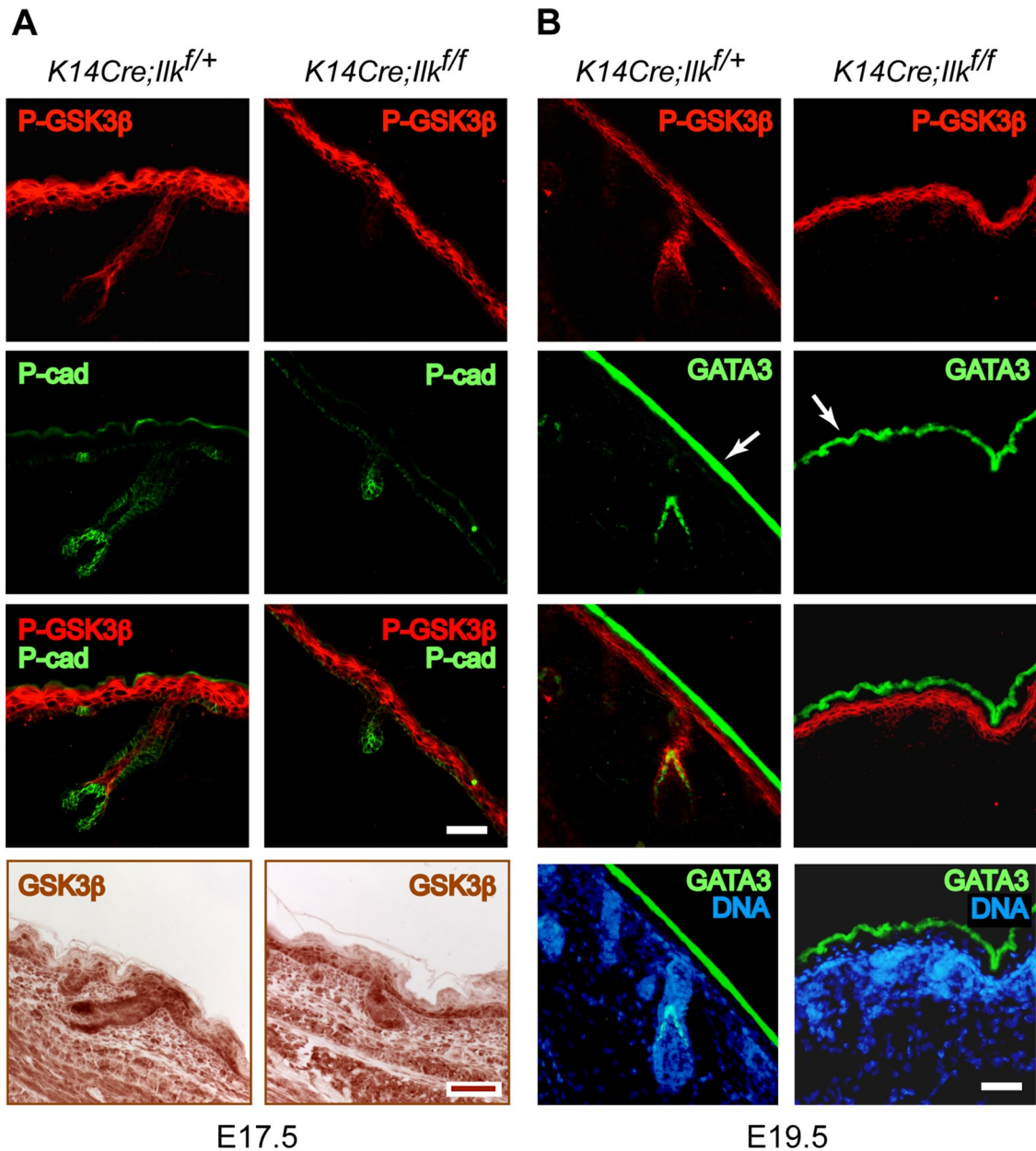


FIGURE 3: Absence of hair matrix-derived cells in ILK-deficient hair follicles. (A) Dorsal tissue sections from E17.5 embryos with the indicated genotypes were processed for immunofluorescence microscopy, using antibodies against GSK3- β phosphorylated at Ser-9 (P-GSK3- β), or for immunohistochemistry, using antibodies against total GSK3- β . Bar, 50 μ m. (B) Dorsal tissue sections from E19.5 embryos with the indicated genotypes were processed for immunofluorescence microscopy, using antibodies against GSK3- β phosphorylated at Ser-9 (P-GSK3- β), or GATA-3. Nuclear DNA was visualized with Hoechst 33342. The arrows indicate antibody trapping in the acellular, upper epidermal layers. Bar, 32 μ m.

filaments were assembled laterally, but very few were observed adjacent to the basement membrane (Figure 4B). In a dissimilar manner, broad F-actin ribbons were apparent all along the outer aspect of the ILK-deficient follicles in contact with the basement membrane in a pattern that resembled that observed for E- and P-cadherin in these cells (Figure 4B). Conversely, integrin β 1 immunoreactivity appeared enriched in regions of ILK-expressing, but not ILK-deficient, hair follicles adjacent to the basement membrane, particularly in those areas juxtaposed to the dermal papilla (Figure 4B). The differences in this pattern of integrin β 1 distribution between ILK-expressing and ILK-deficient follicles were also apparent in E17.5 embryos (unpub-

lished data). Integrin β 1 immunoreactivity was also detected in mesenchymal dermal papilla cells, which were arranged in a highly organized pattern underneath ILK-expressing follicles. In contrast, integrin β 1 staining of dermal papilla cells in ILK-deficient follicles revealed a distinct and disorganized arrangement already at this early stage of development (Figure 4B).

In the interfollicular epidermis, α 6 β 4 integrins form hemidesmosomes in the basal keratinocytes. The α 6 β 4 integrins distribute along cell membrane areas adjacent to the basement membrane and mediate epidermal attachment at the dermal-epidermal junction (Figure 4C; Watt, 2003). In contrast, ILK-deficient interfollicular

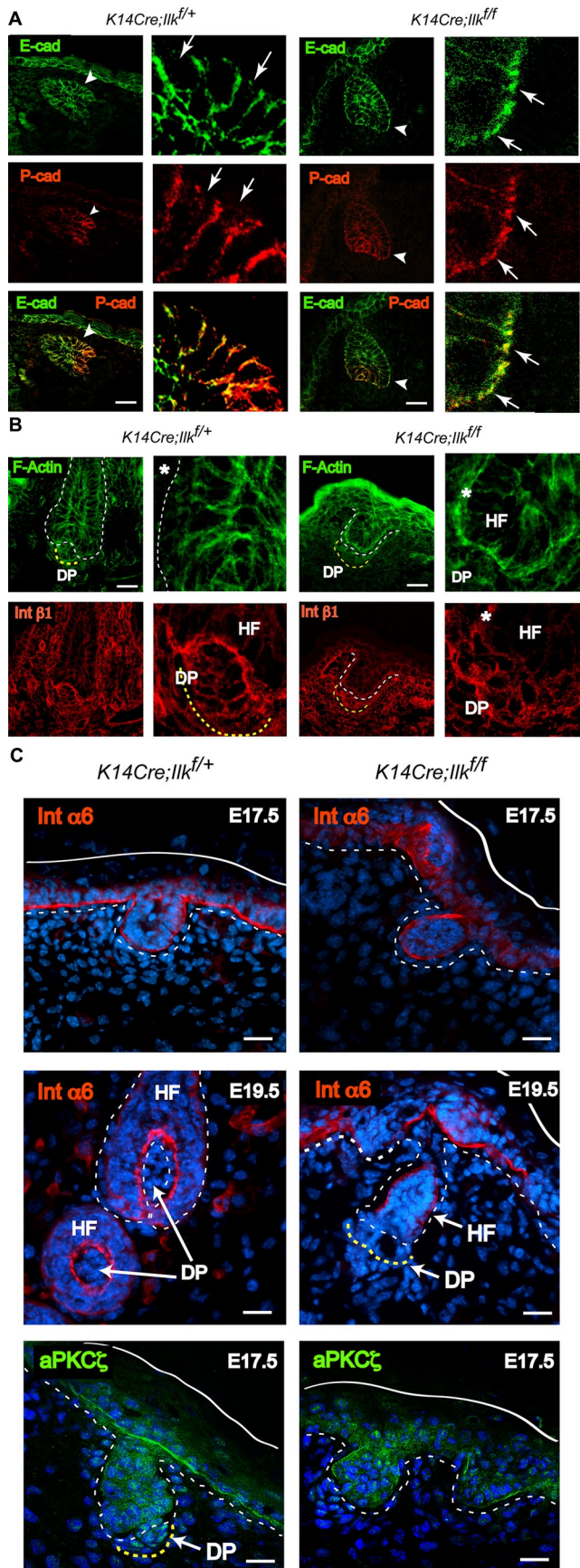


FIGURE 4: Loss of apical–basal cell polarity in ILK-deficient hair follicles. (A) Dorsal tissue sections from E16.5 embryos with the indicated genotypes were processed for confocal microscopy, using

epidermis areas devoid of detectable integrin $\alpha 6$ immunoreactivity are frequently observed in E17.5 embryos (Figure 4C), similar to postnatal epidermis (Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008). Further, in a large fraction of those cells with detectable integrin $\alpha 6$, this protein shows a diffuse, cytoplasmic localization rather than concentration next to the basement membrane. In ILK-expressing hair buds, integrin $\alpha 6$ immunoreactivity is also observed along keratinocyte areas directly adjacent to the basement membrane (Figure 4C). In more developed, E19.5 follicles, integrin $\alpha 6$ also becomes very abundant in matrix keratinocytes, localizing to plasma membrane regions directly in contact with the dermal papilla (Figure 4C). In ILK-deficient hair buds, integrin $\alpha 6$ is also found laterally, juxtaposed to the basement membrane, although areas of discontinuous immunoreactivity are observed. Furthermore, and in contrast to E19.5 ILK-expressing follicles, integrin $\alpha 6$ is not detectable on the ILK-deficient cells at the tip of the developing follicle, which are in direct contact with the dermal condensate (Figure 4C).

In vertebrate epithelia, development of polarity involves the coordinated activation of various pathways, including basal cues from laminins and their receptors, together with the Par complex (Chen and Zhang, 2013; Hohenester and Yurchenco, 2013). The components of the Par complex include Par3, Par6, and atypical protein kinase C (aPKC). In simple epithelia, aPKC isoforms are predominantly found at apical regions and are key for the generation of apical–basal polarity through their role in the formation of tight junctions. In postnatal mouse epidermis, aPKC λ is predominantly found in the cytoplasm of basal keratinocytes and at the cell membrane in suprabasal cells, whereas aPKC ζ appears to be present in the cytosol and the nuclei of basal cells (Helfrich *et al.*, 2007). Given the importance of aPKC in the development of polarity in simple epithelia, we next investigated whether loss of ILK expression affected the distribution of aPKC ζ in embryonic epidermal tissues. In E17.5 ILK-expressing interfollicular epidermis, we readily detected aPKC ζ immunoreactivity in the cytoplasm of basal keratinocytes and also observed enrichment along keratinocyte areas juxtaposed to the basement membrane, in a pattern similar to that described for integrins $\alpha 6$ and $\beta 1$ (Figure 4C). In the hair buds, aPKC ζ was found throughout the cytoplasm and in the nucleus. In contrast, aPKC ζ exhibited a cytoplasmic distribution with no evidence of enrichment at the dermal–epidermal junction in ILK-deficient interfollicular epidermis (Figure 4C). Within the developing ILK-deficient hair buds, we noted aPKC ζ immunoreactivity at borders adjacent to the basement membrane in the upper regions of the hair bud but not in cells directly in contact with the dermal condensate (Figure 4C). Thus, although the patterns of distribution of aPKC ζ in embryonic epidermal epithelia appear to differ from those in other vertebrate epithelia, *Ilk* gene inactivation results in altered localization of this protein in both embryonic interfollicular epidermis and developing hair follicles.

antibodies against E- or P-cadherin. Arrows indicate the basal region of the epithelial cells adjacent to the basement membrane. Areas indicated with an arrowhead are shown at higher magnification in the corresponding adjacent micrographs. (B) Sections prepared as in A were probed with antibodies against integrin $\beta 1$, together with Alexa 488–conjugated phalloidin, to visualize the actin cytoskeleton. The asterisks indicate basal regions of the epithelial cells. (C) Dorsal tissue sections from embryos with the indicated ages and genotypes processed for indirect immunofluorescence or confocal microscopy, using, respectively, antibodies against integrin $\alpha 6$ (Int $\alpha 6$) or aPKC ζ . Nuclear DNA was stained with Hoescht 33342. Solid lines outline the epidermal surface. White and yellow dashed lines in B and C are used to outline, respectively, the hair follicle and the dermal papilla. DP, dermal papilla; HF, hair follicle. Bar, 20 μ m.

A fraction of phenotypic alterations caused by *Ilk* gene inactivation resemble those that arise from loss of integrin $\beta 1$ expression. Hence, to further explore the relationship between ILK and integrin $\beta 1$ during hair follicle morphogenesis, we generated *K14Cre;Intb1^{f/f}* mice with epidermis-restricted inactivation of *Intb1* and compared them with integrin $\beta 1$ -expressing, *K14Cre;Intb1^{f/+}* littermates. Hair follicles in the latter developed normally and showed LEF-1 expression in the epithelial cells adjacent to the dermal papilla, as well as mutually exclusive areas of P-cadherin expression in inner matrix cells and E-cadherin localization to the remainder of the developing hair follicle at stages 4/5 (Figure 5). Integrin $\beta 1$ was not detected in the interfollicular epidermis or in the hair follicles of *K14Cre;Intb1^{f/f}* mice. In these follicles, which appeared arrested around stage 3, as previously reported (Brakebusch *et al.*, 2000; Raghavan *et al.*, 2000), LEF-1 was readily detected in the condensed mesenchyme corresponding to the dermal papilla, but expression in the adjacent epithelium was not uniformly observed in all cells. Further, P- and E-cadherin immunoreactivity was observed throughout the follicle. These alterations in morphology and gene expression are essentially identical to those observed in ILK-deficient follicles, suggesting the possibility that integrin $\beta 1$ and ILK are joint components of a key pathway necessary for hair follicle morphogenesis. Together our data show that the absence of ILK in the developing hair bud results in perturbations in the establishment of apical-basal cell polarity.

Non-cell-autonomous role of epithelial ILK in the dermal papilla

Essential for hair follicle morphogenesis is the reciprocal exchange of signals between the epithelial cells and the mesenchyme that constitutes the dermal papilla. Genome-wide studies on dermal papilla isolates identified multiple transcripts associated with the ability of these mesenchymal cells to induce epithelial downgrowth, and they include versican, CD133, and alkaline phosphatase (Kishimoto *et al.*, 1999; Rendl *et al.*, 2005; Yang and Cotsarelis, 2010). The extracellular matrix protein versican is an important stimulus for dermal papilla cells and is necessary to maintain anagen hair growth (Kishimoto *et al.*, 1999; Yang and Cotsarelis, 2010). In agreement with these properties, we detected versican in dermal papilla cells, with more intense immunoreactivity in postnatal hair follicles (Figure 6). However, versican was not detected in dermal papilla cells of ILK-deficient hair follicles as late as 3 d after birth, when most mutant mice succumb (Figure 6). CD133, a hematopoietic stem cell marker that is also expressed in hair follicles at early stages of development (Yang and Cotsarelis, 2010), and alkaline phosphatase activity were detected in dermal papilla cells, irrespective of the presence or absence of ILK in the epithelium (Figure 6). These observations suggest that, in the absence of ILK, the expression of at least one important dermal papilla marker associated with hair-inducing properties is also disrupted. Further, ILK distributes to the plasma membrane in dermal papilla cells of ILK-expressing, but not ILK-deficient, follicles. These results suggest the possibility that, without epithelial ILK, some aspects of the communication signals between the hair follicle epithelium and the adjacent dermal papilla mesenchyme are altered.

Role of ILK and integrin $\beta 1$ in basement membrane laminin-511 assembly in developing hair follicles

Dynamic regulation of extracellular matrix protein expression is required for normal hair follicle morphogenesis. During hair follicle elongation, a basement membrane preponderantly composed of laminin-511 is assembled surrounding the follicular epithelium. The presence of laminin-511 is essential for hair follicle growth, because

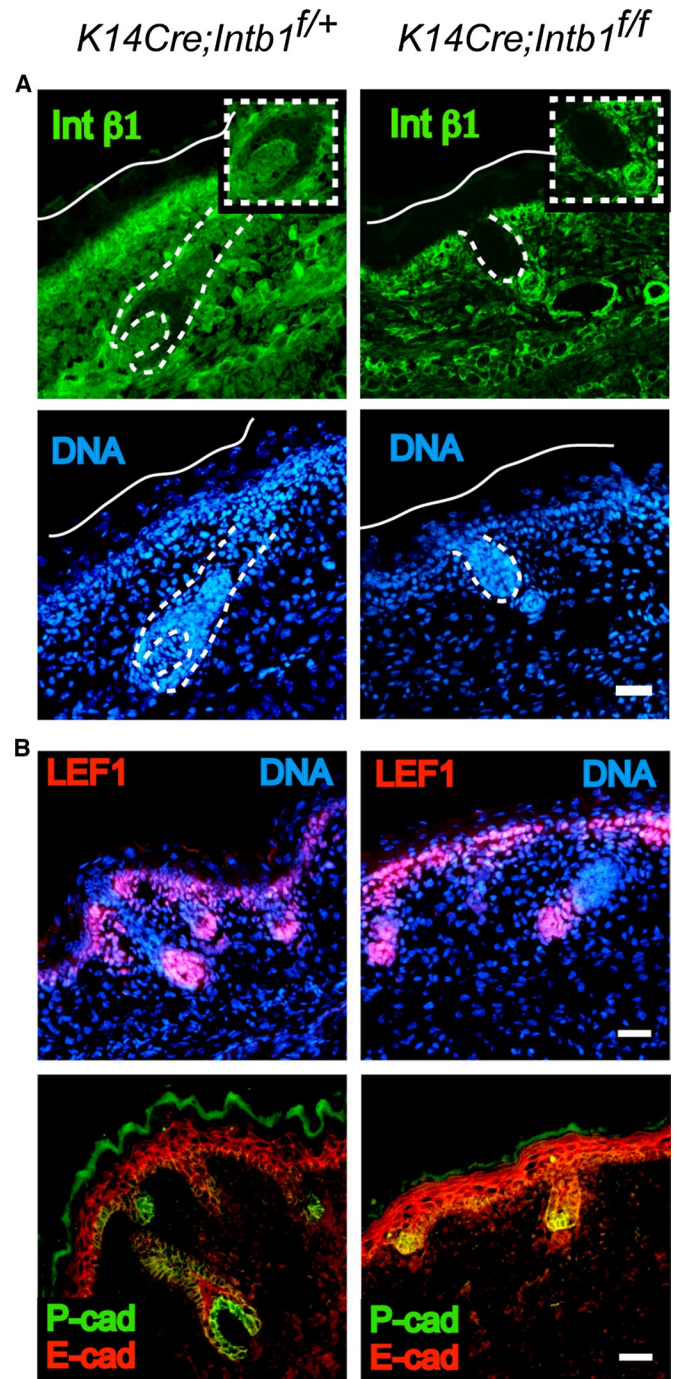


FIGURE 5: Abnormal matrix formation in integrin $\beta 1$ -deficient hair follicles. (A) Dorsal tissue sections from E17.5 embryos with the indicated genotypes were processed for immunofluorescence microscopy, using antibodies against integrin $\beta 1$, or Hoechst 33342 to visualize nuclear DNA. The dashed lines indicate the epithelial cells in the hair follicle, and the arrow shows integrin $\beta 1$ distribution at the junction between the hair matrix cells and the dermal papilla. (B) Dorsal tissue sections from integrin $\beta 1$ -expressing or -deficient E17.5 embryos with the indicated genotypes processed for immunofluorescence microscopy, using antibodies against the indicated proteins. Hoechst 33342 was used to visualize nuclear DNA. Solid lines indicate the surface of the epidermis, and the dashed lines indicate the epithelial cells in the hair follicle. Bar, 32 μ m.

targeted inactivation of *Lama5*, which results in the absence of laminin-511 production, also causes hair follicle development arrest before stage 4 (Li *et al.*, 2003; Gao *et al.*, 2008). Although the exact

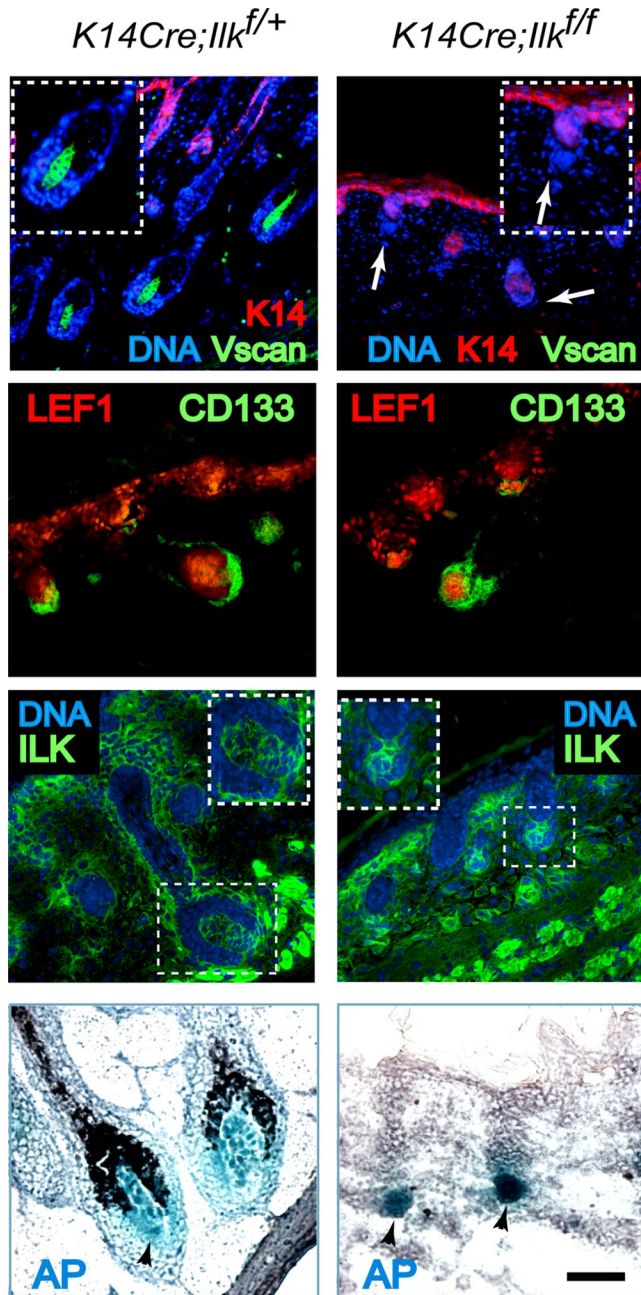


FIGURE 6: Expression of hair-inducing dermal papilla markers in ILK-deficient hair follicles. Dorsal skin sections harvested from mice of the indicated genotypes were processed for immunofluorescence microscopy, with antibodies against keratin 14 (K14), versican (Vscan), LEF-1, CD133, or ILK. DNA was visualized with Hoechst 33342. Alkaline phosphatase (AP) activity in tissue sections was detected using the chromogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate, as described in the Supplemental Materials and Methods. Arrows indicate the dermal papilla in ILK-deficient hair follicles, and insets represent higher-magnification images of boxed areas. Micrographs showing versican and AP staining show tissues from 3-d-old animals, whereas those showing CD133 and ILK were obtained from E17.5 embryos. Bar, 50 μ m.

mechanisms that govern laminin deposition and basement membrane assembly are poorly understood, it has been established that in developing epithelia, laminin assembly into a polymeric network

requires the development of apical–basal cell polarity and the activity of integrins, which also serve as laminin receptors. Reciprocally, proper assembly of the basement membrane is necessary for morphogenetic signaling events that govern epithelial development (Schuger *et al.*, 1998; Klinowska *et al.*, 1999).

The abnormalities in keratinocyte polarity, together with the similarities in hair follicle defects associated with lack of laminin-511 or ILK, prompted us to compare the patterns of laminin-511 immunoreactivity in the basement membrane of ILK-expressing and ILK-deficient hair follicles. Laminin-511 was readily detected all around the hair follicles and in the dermal–epidermal basement membrane zone that links the inner hair matrix with the dermal papilla in both E17.5 and 3-d-old *K14Cre;Ilk^{f/+}* mice (Figure 7A). In ILK-deficient tissues, laminin-511 immunoreactivity was observed on the external aspect of the hair follicle. However, severe disruptions of laminin-511 deposition were evident at the tip of the hair bud, specifically in areas adjacent to the dermal papilla (Figure 7A). This abnormal pattern provides a strong suggestion that laminin-511 is misassembled at the basement membrane. Of importance, similar alterations in laminin-511 deposition were observed in integrin β 1-deficient hair follicles (Supplemental Figure S4), indicating that ILK and integrin β 1 may function in a pathway that regulates the generation of apical–basal polarity in the hair follicle by mechanisms that include proper assembly of the laminin-511 basement membrane.

Given that laminin-511 is a well-established epithelial signal that contributes to the establishment of apical–basal polarity, we reasoned that the observed disrupted pattern of laminin-511 immunoreactivity might contribute to the defects in hair follicle growth in ILK-deficient epidermis. To test this hypothesis, we developed an embryonic explant model in which we first isolated dorsal skin and subcutaneous tissue from E15.5 *K14Cre;Ilk^{f/+}* embryos and cultured them for up to 4 d. Under the culture conditions used, these tissues remained viable and, although the hair follicles in the explants appeared to be slightly shorter than those observed *in vivo*, they developed a well-defined hair matrix surrounding the dermal papilla (Figure 7B). In addition, the cells corresponding to the inner matrix, but not those in the remainder of the follicle, expressed P-cadherin in a pattern analogous to that observed in normal embryonic follicles (Figure 7C).

Analysis of explants from *K14Cre;Ilk^{f/f}* embryos revealed the presence of abnormal follicles lacking a well-defined matrix and expressing P-cadherin throughout all epithelial cells, as observed *in vivo* (Figure 7, B and C). To determine whether exogenously added laminin-511 could restore hair follicle development, we incubated the embryonic explants for 16 h in a solution containing laminin-511 before placing them in culture. This treatment was without effect on ILK-expressing tissues. Significantly, ILK-deficient hair follicles developed morphologically defined structures resembling the hair matrix. Furthermore, laminin-511-treated follicles exhibited a pattern of P-cadherin expression that was limited to a subset of cells directly adjacent to the dermal condensates, and no P-cadherin immunoreactivity was observed in the remaining epithelial cells, in a pattern reminiscent of that observed in ILK-expressing follicles (Figure 7, B and C). Thus the presence of exogenous laminin-511 is capable of restoring formation of follicular hair matrix in the absence of ILK. The results of these experiments are consistent with the notion that ILK participates in the generation of hair follicle matrix keratinocytes by promoting proper assembly of laminin-511 at the interface between hair follicle epithelium and dermal papilla. In this manner, ILK also modulates the development of “apical–basal” keratinocyte polarity, essential for hair follicle morphogenesis.

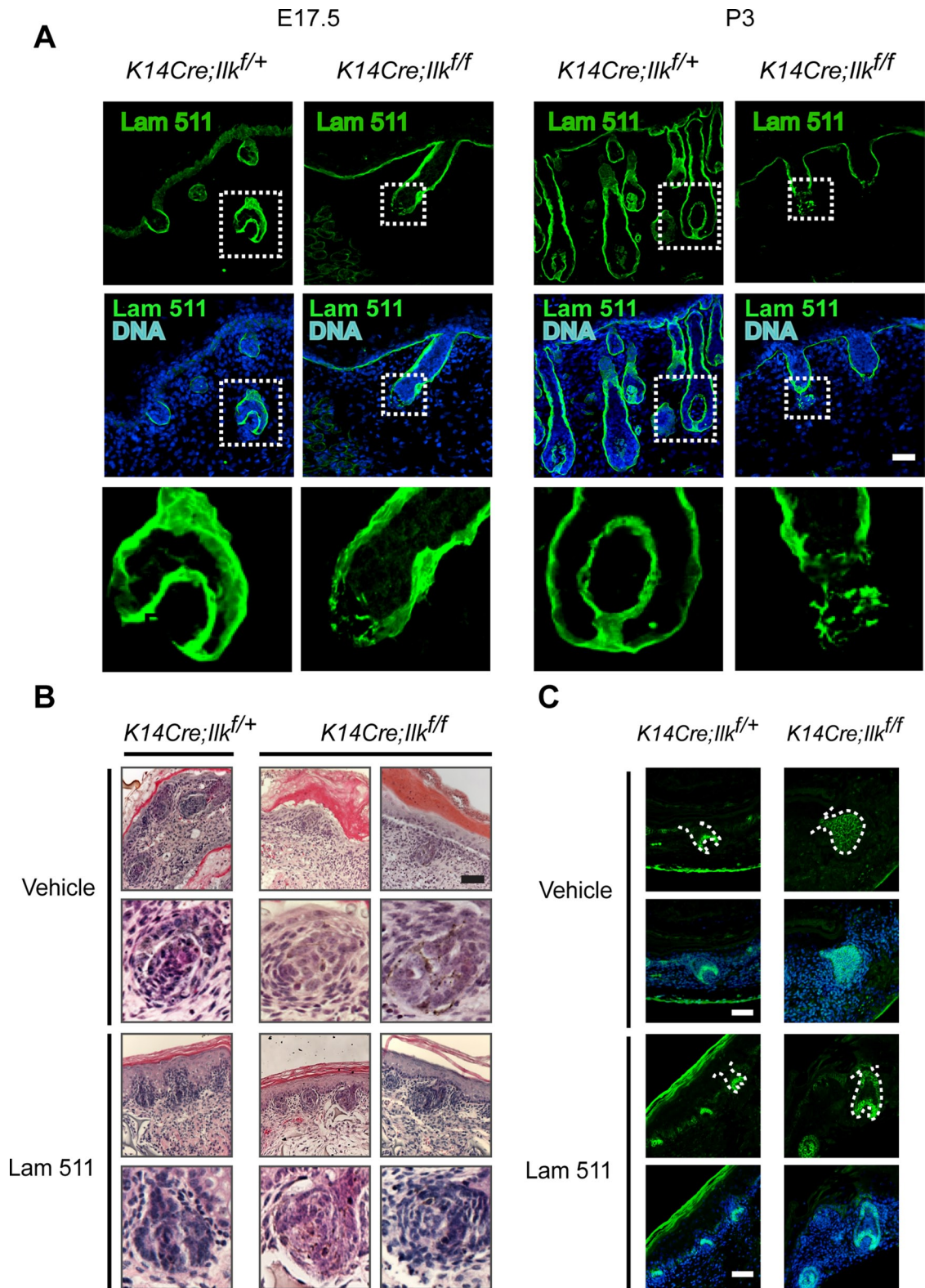


FIGURE 7: Restoration of hair matrix in ILK-deficient skin explants cultured in the presence of laminin-511. (A) Dorsal skin sections harvested from mice of the indicated genotypes and ages were processed for immunofluorescence microscopy, with antibodies against laminin-511. Bottom, boxed areas at higher magnification. DNA was visualized with Hoechst 33342. Bar, 50 μ m. (B) Skin explants from E15.5 embryos of the indicated genotypes were treated with vehicle or laminin-511 for 16 h, followed by culture on polycarbonate inserts for 4 d, as described in *Materials and Methods*. The explants were processed for histology and stained with hematoxylin and eosin. Boxed areas are shown at higher magnification underneath each micrograph. Bar, 32 μ m. (C) Sections from the explants described in B were processed for immunofluorescence microscopy using antibodies against P-cadherin. Nuclear DNA was visualized with Hoechst 33342. Results representative of explants isolated from five to seven embryos (from two or three different litters) for each treatment are shown. Bar, 32 μ m.

DISCUSSION

Multiple inductive signals govern hair follicle morphogenesis. Our studies demonstrate that ILK plays a pivotal role linking the development of apical–basal polarity with morphogenetic responses. Together these processes participate in the specification of hair follicle matrix cells and subsequent formation of the inner root sheath lineage and invagination of hair follicles.

Hair placodes form in response to Wnt signals between ectodermal and neighboring mesenchymal cells through stabilization of β -catenin and LEF-1–mediated transcription of target genes (Millar, 2002; Lee and Tumber, 2012). Sonic hedgehog inductive signals are subsequently generated and are required to maintain proliferation of the epithelial cells in the hair bud, but they appear to be dispensable for the specification of the hair matrix cells at this stage (St. Jacques *et al.*, 1998). In epithelial cells of stage 1/2 hair buds, E- and P-cadherin are coexpressed. As a result of the activation of multiple pathways, further development of the hair bud and specification of hair matrix cells occurs, which coincides with a change in the expression patterns of epithelial cadherins (Muller-Rover *et al.*, 1999; Jamora and Fuchs, 2002; Jamora *et al.*, 2005). Specifically, P-cadherin and E-cadherin are, respectively up- and down-regulated at the leading edge of the hair buds, which subsequently become the matrix cells that surround the dermal papilla. In the remainder of the developing follicle, E-cadherin expression remains elevated. The transcriptional repression of *Cdh1* is essential for hair buds to progress to stages 4/5 and requires TGF- β 2, Wnt/LEF-1, and inhibition of BMP signaling, but it does not appear to involve sonic hedgehog–dependent pathways (Jamora and Fuchs, 2002; Jamora *et al.*, 2005). Our studies now show that the presence of functional cell adhesion platforms containing integrin β 1 and ILK is also essential for the cadherin switch that accompanies the formation of hair matrix cells. Without epithelial ILK, additional alterations that suggest impairment in the maturation and, possibly, in the development of hair-inducing properties in the dermal papilla appear to occur, as suggested by the observation that the arrested hair follicle bud eventually degenerates, as observed in perinatal *K14Cre;Ilk^{fl/fl}* mice.

Although gene inactivation studies provide ample evidence that integrins and their downstream effectors, such as ILK, play key roles in hair follicle development and maintenance (Brakebusch *et al.*, 2000; Raghavan *et al.*, 2000; Lorenz *et al.*, 2007; Nakrieko *et al.*, 2008, 2011), the molecular mechanisms involved have remained virtually unexplored. Through the embryonic characterization of mice in which epidermis-restricted *Ilk* gene inactivation is induced well before initiation of folliculogenesis (Dassule *et al.*, 2000; Nakrieko *et al.*, 2008), we now provide evidence of a crucial role for ILK in the specification of matrix and inner root sheath hair follicle cell lineages. Significantly, it would appear that ILK is dispensable for the formation of matrix and inner and outer root cell lineages once these early stages of follicle development have taken place. This is consistent with the observation that conditional *Ilk* gene inactivation in the epidermis induced by expression of Cre recombinase around E15.5, reported in a different mouse line (hereafter termed ILK-K5 mice), appears to have little, if any, effect on the formation of hair follicles as late as stage 8 (Lorenz *et al.*, 2007).

Our results are also consistent with a role for ILK as a central regulator of apical–basal cell polarity in hair buds, which, in turn, plays key roles in further development of the hair follicle. Accordingly, *Ilk* gene inactivation gives rise to pronounced alterations in the distribution of integrins α 6 and β 1 and E- and P-cadherin along plasma membrane domains of follicular keratinocytes in E16.5–19.5 embryos. Such alterations precede failure of hair matrix cell specification, generation of inner root sheath cells, and additional follicle

growth and invagination. These alterations further contrast with the reported normal apical–basal polarity and presence of matrix and inner root sheath cell markers in ILK-K5 mice (Lorenz *et al.*, 2007). Our data also demonstrate that the absence of appropriate apical–basal polarity in ILK-deficient follicular hair bud cells accompanies altered laminin-511 assembly and basement membrane organization. This phenomenon may be caused, at least in part, by the abnormal distribution of β 1 integrins, which are required for proper laminin assembly (Hamill *et al.*, 2009). To determine whether developmental arrest in ILK-deficient follicles is a downstream consequence of laminin misassembly, we investigated the ability of exogenously provided laminin-511 to restore hair matrix formation in cultured skin explants. Strikingly, exogenous laminin-511 was able to induce hair matrix formation and proper distribution of P-cadherin in developing follicles without affecting the development of ILK-expressing follicles. These observations are in agreement with the reported ability of exogenous laminin-511, but not laminin-332, to restore hair follicle development in laminin-511–deficient epidermis (Gao *et al.*, 2008). The observation that exogenous laminin-511 is sufficient to rescue hair matrix formation implies a causal link between ILK deficiency, laminin misassembly, and hair follicle growth arrest at this early stage. It is also consistent with the notion that ILK is required for basement membrane assembly but not for subsequent laminin signaling to the cell, at least during this stage of development. Of note, the role of ILK in regulation of apical–basal polarity in hair buds differs from that in mammary gland development, as ILK is also necessary to mediate processes downstream from cell activation by laminins in mammary gland tissues (Akhtar and Streuli, 2013).

Another molecular consequence of the disruptions caused by early loss of ILK is the impaired responses to Wnt signaling in the stage 2/3 hair follicles. Specifically, alterations in LEF-1 and E- and P-cadherin, as well as in the pattern of distribution of phosphorylated GSK-3 β , occurred in ILK-deficient follicles. This is associated with impaired specification of hair matrix cells, which mature and give rise to the precortex and the inner root sheath, characterized by the presence of E-cadherin, GATA-3, and phosphorylated GSK3- β . This disruption contrasts as well with the apparently normal LEF-1–dependent processes reported in ILK-K5 mice (Lorenz *et al.*, 2007), further underlining the spatiotemporal character of various ILK functions during epidermal development. Of note, ILK likely modulates other laminin-511–independent pathways, as some alterations observed in ILK-deficient follicles do not appear to be recapitulated in laminin-511–deficient mice.

Epithelial cells can form apical–basal or front–rear polarity axes, the latter of which are involved in two-dimensional cell migration. A major challenge in biology is to understand how cells regulate processes that generate different types of asymmetry, which allow them to acquire a variety of specialized functions. We now show that, in addition to its importance in the development of keratinocyte front–rear polarity (Ho and Dagnino, 2012b), ILK is also a central regulator of apical–basal polarity during formation and growth of the hair follicle *in vivo*. Our studies suggest the presence of an autocrine loop in which hair follicle cells create their own polarity cues. On that basis, we propose a model in which ILK regulates the asymmetric distribution of β 1 integrin. Together ILK and the α β 1 integrin pool that localizes to the basal aspect of the cell regulate laminin-511 matrix assembly. Assembled laminin-511, in turn, stimulates the cell to direct the orientation of the apical–basal axis, which also allows responses to other signals (such as Wnt), specification of the hair matrix cell lineage, and epithelial communication with the dermal papilla. Because exogenously provided laminin-511 restores matrix

formation in the absence of ILK, it would appear that ILK is necessary for laminin-511 secretion and/or assembly at the tip of the hair bud, but it is not essential to transduce laminin-511 signals. Besides integrin $\beta 1$, several receptors for laminin-511 have been identified, including heparan sulfate and dystroglycan, and it is possible that these molecules mediate responses to appropriately assembled laminin-511 during hair matrix formation. In short, our data place ILK as a hub for signaling processes that integrate multiple inputs within the complex microenvironment that surrounds the developing hair follicle epithelium. Significantly, major defects in ILK-deficient hair buds appear to occur in areas adjacent to the dermal condensate, which give rise to matrix cells.

The biochemical events that occur downstream from ILK and result in loss of cell polarity may also involve improper GTPase activation. Indeed, cultured ILK-deficient keratinocytes show impaired Rac1 activation in response to various stimuli (Ho *et al.*, 2009; Sayedyahosseini *et al.*, 2012), and Rac1 has been implicated in the establishment of apical-basal epithelial cell polarity and hair follicle formation (O'Brien *et al.*, 2001). Clearly, this will be an important aspect for future research.

MATERIALS AND METHODS

Mice and skin grafting experiments

The generation and characterization of mice with epidermis-restricted inactivation of *Ilk* or *Intb1* have been described (Nakrieko *et al.*, 2011; Ho and Dagnino, 2012b; Sayedyahosseini *et al.*, 2012). For experiments with embryonic tissues, midday of the day on which vaginal plugs appeared was considered as E0.5 for timed pregnancies. All results shown are representative of analyses conducted on multiple tissue sections obtained from three to six animals per genotype and were littermates generated from two to five litters.

Female Crl:NU-Foxn1^{nu} immunodeficient mice (8 wk old) were used as graft recipients. For transplant experiments, 3-d-old *K14Cre;Ilk^{fl/+}* mice and *K14Cre;Ilk^{fl/fl}* littermates were killed, and immediately thereafter sections of full-thickness dorsal skin (1.5 × 1.5 cm) were harvested, spread, and stored briefly on a culture dish at 4°C. After harvesting each skin, the anesthetized graft recipient was prepared by removing a patch of full-thickness dorsal skin. The donor skin was placed onto the graft bed, covered with antibiotic ointment, and secured with sterile gauze and cloth bandages. The genotype of each donor was confirmed from tissue samples obtained at the time of tissue harvesting, as described (Nakrieko *et al.*, 2011; Ho and Dagnino, 2012b; Sayedyahosseini *et al.*, 2012). A total of five *K14Cre;Ilk^{fl/+}* and five *K14Cre;Ilk^{fl/fl}* grafts were transplanted. All the grafts of a given genotype showed consistent phenotypes. All animal experiments were approved by the University of Western Ontario Animal Use Care Committee in accordance with regulations and guidelines from the Canadian Council on Animal Care.

Immunohistochemistry, immunofluorescence, and light microscopy

For immunohistochemical detection of Ki67 or total GSK-3 β , 7- μ m paraffin-embedded sections were subjected to high-temperature antigen retrieval, with 10 mM sodium citrate (pH 6.0), followed by incubation with primary antibody, using Vectastain Elite ABC kits and ImmPRESS reagent (Vector Laboratories, Burlingame, CA). Immunoreactivity signals were visualized with ImmPACT NovaRED peroxidase substrate (Vector Laboratories), and tissues were counterstained with methyl green. To detect versican and LEF-1, embryos were fixed in Zn buffer (0.1 M Tris-HCl, pH 7.8, 0.05% calcium acetate, 0.5% zinc acetate, 0.5% zinc chloride) and 4% paraformaldehyde, respectively, embedded in paraffin, and sectioned (7 μ m).

Tissues were subjected to antigen retrieval before incubation with primary antibodies. To detect all other proteins, embryos were protected by embedding in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) before freezing. Cryosections (7–10 μ m) were fixed either with acetone:chloroform (1:1 vol/vol, –20°C, 15 min) to detect ILK or phospho-GSK3- β or with 4% paraformaldehyde for all other proteins. Fixed tissues were treated for 15 min with 0.2% Triton X-100 in phosphate-buffered saline (PBS), followed by a 30-min blocking incubation with Powerblock Universal Blocking Reagent (HK085-5K; BioGenex, San Ramon, CA). M.O.M. basic kits (Vector Laboratories) were used with mouse monoclonal primary antibodies, following the manufacturer's instructions. Alkaline phosphatase activity in frozen, paraformaldehyde-fixed tissue sections was detected using BM Purple AP (11971100; Roche, Indianapolis, IN).

The determination of developmental stages in hair follicles was conducted according to previously established criteria (Paus *et al.*, 1999). Fluorescence photomicrographs were acquired with a Leica DMIRBE microscope (Leica, Wetzlar, Germany) equipped with an Orca-ER digital camera (Hamamatsu Photonics, Hamamatsu, Japan), using Volocity 4.3.2 software (Improvision, Coventry, United Kingdom). Light microscopy images were obtained with a Zeiss Axio Imager Z1 microscope equipped with a Zeiss AxioCam ICC 1 camera, using Axio Vision 4.6.3 software (Carl Zeiss, Thornwood, NY). Confocal analysis was conducted with a Zeiss LSM 510 DUO scanning laser confocal microscope, using ZEN 2009 SP1 software (Zeiss, Jena, Germany).

Immunoblot analysis

Protein lysates from dorsal skin were prepared by homogenizing 60–70 mg of tissue in lysis buffer A (100 mM Tris-HCl, pH 6.8, 1% NP-40, 10 mM EDTA, 1 M urea). Epidermal lysates were generated by initial incubation of skin in Dispase II (8 mg/ml, final; Roche) for 1 h at 37°C. After digestion, the epidermis was mechanically separated from the dermis and homogenized in lysis buffer A. Lysates were resolved by denaturing PAGE and analyzed by immunoblot, as described (Dagnino *et al.*, 2010).

Embryonic skin explant cultures

Embryonic skin explant cultures were established as reported (Chang *et al.*, 2004), with some modifications. Embryos at E15.5 were harvested, and the dorsal skin was isolated, minimizing the contribution of underlying mesenchymal tissues. The tissue was spread on a sterile gelatin sponge (Gelfoam; Upjohn, Kalamazoo, MI), with the epidermis side up. The sponges were immersed in PBS in the presence or absence of purified laminin-511 (40 μ g/ml, final) and incubated at 4°C for 16 h. The sponges were then transferred onto 24-mm polycarbonate inserts (8- μ m pore size) in a two-chamber culture system (3428, Costar Transwell; Corning, Corning, NY) in which only the bottom chamber contained 2 ml of DMEM, supplemented with 2% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μ g/ml). The explants were cultured at 37°C for 4 d, with medium changes every 48 h. At the end of the culture period, the tissues and sponges were fixed in 4% paraformaldehyde and embedded in paraffin. We used 7- μ m sections for histology and immunofluorescence microscopy. Each experimental condition was evaluated on explants from five to seven embryos isolated from four different litters.

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