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Planar cell polarity-dependent and independent functions in the emergence of tissue-scale hair follicle patterns

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ABSTRACT

Hair follicles of the mammalian epidermis display local order and global alignment, a complex pattern instructed by the core planar cell polarity (PCP) pathway. Here we address the contributions of core PCP genes, Van Gogh-like and Frizzled, to the establishment, local refinement, and global order of embryonic and postnatal hair follicles. We find that, similar to Fz6 mutants, the disordered hair patterns of Vangl2 mutants are refined over time and eventually corrected. In both mutants, we find that tissue-level reorientation occurs through locally coordinated follicle rotation at stereotyped locations. Strikingly, Vangl2 and Fz6 mutant follicles collectively rotate with opposing directionalities, suggesting that redundant core PCP signals contribute to their directed realignment. Consistently, global follicle alignment is not restored upon conditional ablation of both Vangl1 and Vangl2 genes. Instead, spatially distinct patterns of whorls and crosses emerge and persist even after a complete cycle of hair follicle regeneration. Thus, local refinement of hair follicles into higher order patterns can occur independently of the core PCP system, however, their global alignment with the body axes requires PCP function throughout morphogenesis, growth and regeneration.

1. Introduction

The skin of mammals is decorated and protected by a coat of highly ordered body hairs that point in a uniform orientation over the skin surface. Mammalian hairs derive from hair follicles, complex multicellular miniorgans that produce hair and regenerate throughout life. Each hair follicle in its fully developed form is comprised of hundreds of epithelial cells organized in concentric layers that are embedded within and ensheathed by stromal cells of the underlying dermis (Schneider et al., 2009). Although hair follicles are spatially separated from one another by interfollicular epithelial cells and dermal fibroblasts, they display remarkable local coordination and global order, representing a striking yet complex example of planar cell polarity (PCP) (Devenport and Fuchs, 2008; Guo et al., 2004). While it is known that a highly conserved 'core' PCP pathway acts during embryogenesis to direct the polarity pattern of murine hair follicles, the temporal and spatial requirements of core PCP genes during hair follicle morphogenesis, growth and postnatal cycling remain unclear (Chang et al., 2016; Devenport and Fuchs, 2008; Wang et al., 2010). Here we address the contributions of the core PCP genes, Van Gogh-like and Frizzled, to the establishment, local refinement, and global order of embryonic and postnatal hair follicles.

PCP orients diverse cellular structures across a wide range of tissues and organisms. It is best understood in simple epithelia such as the *Drosophila* wing blade where PCP orients polarity at the level of individual cells (Adler, 2012; Devenport, 2016). Based on studies primarily in the *Drosophila* wing, it is generally understood that membrane-associated core PCP proteins asymmetrically localize at cell junctions where they interact intercellularly to form asymmetric bridges. Frizzled (Fz) and Flamingo (Fmi, Celsr in vertebrates) on one side of the junction interact with Van Gogh (Vang, Vangl in vertebrates) and Fmi on the opposing interface (Usui et al., 1999; Lawrence et al., 2004; Chen et al., 2008; Strutt and Strutt, 2008; Wu and Mlodzik, 2008; Struhl et al., 2012). Intracellularly, Vang and Fz proteins act antagonistically through their cytoplasmic binding partners to reinforce polarity within a cell. Global, tissue-level directional cues bias the asymmetric localization of core PCP proteins, which in turn act on downstream cytoskeletal and trafficking effectors to generate the structural polarity of individual cells (Devenport, 2014; Goodrich and Strutt, 2011; Vladar et al., 2009). How these principals apply to much more complex multicellular structures, like mammalian hair follicles, is poorly understood.

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Developing hair follicles gain their first signs of asymmetry during embryogenesis, shortly after hair follicle placodes first invaginate into the underlying dermis (Devenport and Fuchs, 2008). Hair follicle placodes form an acute and unidirectional tilt that is oriented in an anterior direction over most of the body surface. Proliferation drives further anterior-directed growth as follicles maintain and refine their anterior-biased position. Following differentiation into several concentric and specialized cell layers, hair follicles produce keratin-rich hairs that protrude from the skin surface in a posterior direction (Schneider et al., 2009). Follicles then undergo several rounds of destruction (catagen) and regeneration (anagen) throughout postnatal life (Alonso and Fuchs, 2006), yet maintain their precise and ordered planar polarized pattern. Whether continued input from the core PCP pathway is required to maintain and refine the postnatal hair pattern is not known.

The core PCP genes Vangl2, Celsr1, and Fz6 are required for hair follicle alignment where they act during early stages of hair follicle morphogenesis to drive the anterior-directed tilt of hair follicle placodes (Chang et al., 2016; Devenport and Fuchs, 2008; Guo et al., 2004; Ravni et al., 2009; Wang et al., 2010). Prior to hair follicle downgrowth, Vangl2, Celsr1, and Fz6 proteins become asymmetrically localized within basal epidermal cells – progenitor cells that give rise to hair follicles (Fig. 1A; Devenport and Fuchs, 2008). Vangl2 preferentially localizes to the anterior side of basal cells, while Fz6 becomes enriched on the posterior. Celsr1, an atypical cadherin, localizes to both anterior and posterior sides, where it is thought to bridge Vangl2 and Fz6 between adjacent cells. PCP protein asymmetry prefigures the orientation of hair follicles, which tilt and elongate in the direction of Vangl2 localization (Devenport and Fuchs, 2008; Devenport et al., 2011). In the absence of Vangl2 or Celsr1 function, hair placodes invaginate perpendicular to the epithelium and point straight downward, whereas Fz6 mutant follicles emerge at random orientations (Chang et al., 2016; Devenport and Fuchs, 2008; Wang et al., 2010).

During postnatal stages, hair follicle angles become increasingly coordinated, both locally and globally, to align precisely with the anterior-posterior axis. Strikingly, this process of postnatal refinement occurs independently of Fz6 function (Wang et al., 2006, 2010). In Fz6 KO mice, the initially disordered pattern of hair follicle angles corrects over time, whereby at postnatal day 21, the normal hair follicle pattern is completely restored (Chang and Nathans, 2013). The ability of hair follicles to rotate within the dermis and correct their orientations highlights remarkable plasticity of adult hair follicles. However, hair follicle refinement has not been observed in any other core PCP mutant to date, raising the question of whether this phenomenon is unique to the Fz6 allele. There are over 10 different Fz homologs present in the mouse genome, and deletion of Fz6 produces only mild defects compared to other PCP mutants (Wang et al., 2016). Thus, it is unclear whether redundant core PCP cues instruct the postnatal hair follicle pattern in Fz6 mutants, or whether a parallel PCP system, such as the Fat-Dachsous- Four-jointed system (Matis and Axelrod, 2013), functions postnatally to correct hair follicle alignment.

Here, by characterizing tissue-wide patterns of cellular and hair follicle polarity, we define the core PCP-dependent and independent events in the establishment and refinement of the mammalian hair pattern. We find that, although Vangl2 and Fz6 are required for the initial polarization and alignment of nascent hair follicles, the disordered hair patterns of both PCP mutants are refined and eventually corrected, through stereotyped and locally coordinated rotational patterns. Strikingly, Vangl2 and Fz6 mutant follicles collectively rotate with opposing directionalities, suggesting that realignment occurs through redundant core PCP signals. Surprisingly, follicle rotation is not prefigured by PCP protein asymmetry in the interfollicular epidermis. Finally, eliminating all Vangl function through conditional ablation of both Vangl1 and Vangl2 genes in the skin yields a distinct global hair pattern of highly reproducible whorls and crosses that persists during follicle growth and regeneration. These results demonstrate that the local, collective rotation of hair follicles into higher order patterns is a largely PCP-independent process. However, the directional sensing mechanisms that enable follicles to align with the global body axes require continued input from the core PCP pathway.

2. Methods

2.1. Mouse lines and breeding

All procedures involving animals were approved by Princeton University's Institutional Animal Care and Use Committee (IACUC). Mice were housed in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. Please see Table 1 for full genotypes.

2.2. Embryo preparation and whole-mount immunostaining

For embryonic immunostaining, embryos were dissected in PBS and fixed in 4% paraformaldehyde. E15.5 embryos were fixed for 1 h and e17.5 embryos were fixed for 2 h following decapitation. Dissected back skin was blocked for 1 h at room temperature or overnight at 4 °C in 2% normal goat serum, 2% normal donkey serum, 1% bovine serum albumin and 1% fish gelatin in PBT2 (PBS with 0.2% Triton X-100). Skins were incubated with primary antibodies in blocking solution overnight at 4 °C. Skins were washed in PBT2 and incubated with secondary antibodies for 2 h at room temperature or overnight at 4 °C in PBT2. Samples were mounted in Prolong Gold. The following primary antibodies were used: guinea pig anti-Celsr1 (1:1000, D. Devenport), rat anti-E-Cadherin (1:1000, DECMA-1, Thermo Pierce), rat anti-Vangl2 (1:100, 2G4, Millipore), rabbit anti-Vangl2 (1:500, Millipore, 2668504) (pan-Vangl) (Belotti et al., 2012). Alexa Fluor-488,-555, and -647 secondary antibodies were used at 1:1000. Images were acquired on an inverted Nikon A1 or A1R-Si confocal microscope controlled by NIS Elements software using a Plan Apo 60/1.4NA or 40/1.3NA oil immersion objective. ImageJ and Photoshop were used for image processing.

2.3. Quantification of embryonic hair follicle orientation

Full embryonic back skin images were acquired by tiling 20x images obtained on a Nikon Eclipse Ti epifluorescence microscope using a Plan Apo 20/0.75NA objective. NIS Elements software was used to tile and measure follicle angles. A reference angle was set along the anterior posterior axis and a line was drawn over each follicle towards its base. The difference between the two angles provides the orientation of the hair follicle.

2.4. Sample preparation and quantification of postnatal hair follicle orientation

Skin was prepared as previously described (Chang et al., 2014). After euthanasia, animals over 7 days old were shaved and hair was removed using Nair. Dorsal back skin was dissected from P2-P39 animals and pinned to solidified paraffin in a petri dish with the dermis facing up and fixed overnight at 4 °C. Fixed skins were washed in PBS and dehydrated over consecutive days in 70%, 95%, and 100% ethanol. Dehydrated skins were placed between microscope slides in a glass petri dish to keep them flat. BABB (2:1 ratio of benzyl benzoate and benzyl alcohol) was added to clear the tissue overnight. Brightfield images were acquired on a Nikon SMZ1270 dissecting scope using a Plan Apo 0.5x objective and a Nikon Digital Sight Fi1 camera. Samples were illuminated from below the dish and images were taken with 3-8x magnification. Whole backskin images were obtained by stitching together high magnification images with Photomerge in Photoshop.

To account for differences in animal size, each high magnification image was cropped to the same anterior-posterior and lateral region