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Epidermal Wnt controls hair follicle induction by orchestrating dynamic signaling crosstalk between the epidermis and dermis

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Abstract

A signal first arising in the dermis to initiate the development of hair follicles has been described for many decades. Wnt is the earliest signal known to be intimately involved in hair follicle induction. However, it is not clear whether the inductive signal of Wnt arises intradermally or intraepidermally. Whether Wnt acts as the first dermal signal to initiate hair follicle development also remains unclear. Here, we report that Wnt production mediated by *Gpr177*, the mouse *Wls* orthologue encoding a Wnt trafficking regulator, is essential for hair follicle induction. Cell-type specific abrogation of the signal reveals that only epidermal, but not dermal, production of Wnt is required. An intra-epidermal Wnt signal is necessary and sufficient for hair follicle initiation. But, the subsequent development depends on reciprocal signaling crosstalk of epidermal and dermal cells. Wnt signals within the epidermis and dermis, and crossing between the epidermis and dermis, have distinct roles and specific functions in skin development. This study not only defines the cell type responsible for Wnt production, but also reveals a highly dynamic regulation of Wnt signaling at different steps of hair follicle morphogenesis. Our findings uncover a mechanism underlying hair follicle development orchestrated by the Wnt pathway.

Keywords

Gpr177; Wntless; β-catenin; hair placode and skin

Introduction

The mammalian skin and its appendages are derived from ectoderm and mesoderm during embryogenesis (Fuchs, 2007; Hardy, 1992). The embryo surface emerges as a single layer of epithelial cells which give rise to the epidermis. The dermis is formed from the underlying

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Experimental details are described in Supplemental Information

Conflict of Interest

The authors state no conflict of interest

mesoderm composed of mesenchymal cells. Subsequently, the epidermal-dermal interaction results in hair follicle formation (Fuchs, 2007; Hardy, 1992). Morphologically, the development of hair follicles begins with a local thickening of the epidermis, known as hair placodes. Upon successful initiation of the epithelial placode, a condensate of dermal cells is formed in the underlying mesenchyme (Driskell et al., 2011; Fuchs, 2007). The signals sent between this mesenchymal condensate and the overlying epithelial placode dictate the behavior of both cell populations, ultimately orchestrating formation of the hair follicle and dermal papilla (Fuchs, 2007; Hardy, 1992; Millar, 2002; Schmidt-Ullrich and Paus, 2005). Classical cross-species experiments indicate that mouse dermal tissue is capable of inducing the hair placode, feather bud or scale placode upon epithelial-mesenchymal recombination (Dhouailly, 1973; Hardy, 1992; Olivera-Martinez et al., 2004). The data thus suggest existence of an inducing factor arising initially in the dermis. However, the first dermal signal initiating the developmental programming of the epidermis remains elusive.

Several families of secreted signaling molecules have been implicated in the communication between epidermis and dermis during hair follicle development. Among them, the Wnt family appears to be the earliest and most critical regulator for early development of the epidermis (Fuchs, 2007; Millar, 2002; Noramly et al., 1999; Schmidt-Ullrich and Paus, 2005). Wnt blocks the ability of the ectoderm to respond to FGF. This in turn elevates BMP, leading to fate determination of the epidermis (Fuchs, 2007; Stern, 2005). During formation of the hair follicles, multiple Wnt proteins are expressed in the embryonic skin (Millar et al., 1999; Reddy et al., 2001). Activation of Wnt/β-catenin signaling is evident in both the epithelium and the underlying mesenchyme (Andl et al., 2002; DasGupta and Fuchs, 1999; Zhang et al., 2008). Deletion of β-catenin in the epidermis impairs the formation of hair placodes (Huelsken et al., 2001). β-catenin signaling is necessary and sufficient to initiate hair follicle development (Andl et al., 2002; Gat et al., 1998). Although the importance of Wnt signaling in hair follicle development is well established, it is not clear whether the Wnt signal arises intradermally or intraepidermally. Whether Wnt acts as the first dermal signal to initiate hair follicle development also remains to be determined.

Disruption of Wnt production in the signal-producing cells can yield important insights into the mechanism underlying hair follicle development. However, due to overlapping expression of *Wnts* in the developing skin, gene specific inactivation may not be practical, and is likely to encounter issues related to functional redundancy. We have recently identified *Gpr177* as the mouse orthologue of *Drosophila Wls/Evi/Srt* essential for proper sorting and secretion of Wnt (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2011; Fu et al., 2009; Goodman et al., 2006). In flies, Wls/Evi/Srt regulates the secretion of all Wnts, except for WntD, due to its exclusion from lipid modifications (Ching et al., 2008; Herr and Basler, 2012). In mice, genetic studies have suggested that the Gpr177-mediated regulation of canonical and noncanonical Wnts is required for different cell types and tissues (Fu et al., 2011; Fu et al., 2009; Stefater et al., 2011). The abrogation of Wnt secretion caused by Gpr177 deficiency may provide an excellent strategy to determine the source of Wnt during organogenesis. To decipher Wnt signaling regulation in hair follicle development, we created mouse models with cell-type specific disruption of Gpr177. This study not only defines the cell type responsible for Wnt production, but also reveals a highly

dynamic regulation of Wnt signaling, at various steps of hair follicle development. Our findings suggest a model for the role of Wnt signaling in hair follicle morphogenesis.

Results

We examined the expression of Gpr177 to determine the cell type potentially responsible for Wnt production during hair follicle induction. Using a well-characterized antibody specifically recognizing Gpr177 (Fu et al., 2009), immunostaining analysis revealed that Gpr177 is expressed in the epithelium and the underlying mesenchyme at E11.5 and E13.5 (Fig. 1a, b). In the epithelium, Gpr177 expression was restricted to the developing hair follicles and the basal layer of epidermis at E15.5 and E17.5 (Fig. 1c, d). Co-labeling of Gpr177 with AE3, a marker for the entire epidermis, or K14, a marker for the epidermal basal layer, further indicated that the epidermal basal cells and the hair follicular cells express high levels of Gpr177 (Fig. 1e-l and Fig. S1).

To determine the requirement of Gpr177 in the epidermis, we generated $Gpr177^{K5}$ mutant mice in which *Gpr177* was inactivated by K5-Cre (Ramirez et al., 2004). Using the R26R reporter allele, β-gal staining of the E13.5 and E14.5 skins showed the effectiveness of Cre recombination in the epidermis and developing hair follicles (Fig. 2a, b). Immunostaining for Gpr177 further indicated its successful ablation in the epidermis, but not dermis, of $Gpr177^{K5}$ (Fig. 2c, d). Wnt secretion assays further examined epidermal Wnt production affected by the Gpr177 deletion. Primary epidermal cells of control and $Gpr177^{K5}$ were used as the signal-producing cells, which were co-cultured with the signal-receiving cells harboring a TOPFLASH reporter for β-catenin and Lef/Tcf-dependent transcription. While the control epidermal cells were capable of activating the TOPFLASH reporter, this activation was significantly reduced and close to the background level in the $Gpr177^{K5}$ dermal cells (Fig. S2a). Furthermore, activation of several Wnt signaling mediators was affected in the epidermis of $Gpr177^{K5}$ (Fig. S2b). These results suggest that Gpr177 is essential for Wnt secretion in the epidermis. No hair placodes and follicles were detected in the $Gpr177^{K5}$ mutants at E14.5 and E17.5, respectively, indicating an essential role of Gpr177 in the epidermis for hair follicle induction (Fig. 2e-h; 100%, n=15). Next, we examined the expression of placode/follicle-specific genes affected by the loss of Gpr177 (Botchkarev and Sharov, 2004; Laurikkala et al., 2002; Zhang et al., 2009). No expression of ectodysplasin receptor (Edar), BMP2, BMP4 and Shh was found, indicating no formation of the placodes in the $Gpr177^{K5}$ mutants (Fig. 2i-p). The results imply that epidermal Gpr177 is involved in the regulation of Wnt, the earliest developmental signal known to turn on the programming for hair follicle induction (Fuchs, 2007; Millar, 2002; Noramly et al., 1999; Schmidt-Ullrich and Paus, 2005).

Multiple members of the Wnt family were expressed in distinct patterns in the developing skin with a few candidates, e.g. Wnt3, Wnt10a and Wnt10b, implicated in hair follicle morphogenesis (Andl et al., 2002; Millar et al., 1999; Reddy et al., 2001). We first examined the expression of all 19 mouse *Wnt* genes in the E14.5 skin by RT-PCR analysis, and detected transcripts of *Wnts2, 3, 4, 5a, 6, 7a, 7b, 10a, 10b, 11* and *16*, including those reported (Reddy et al., 2001), whose expression patterns were further characterized (Fig. S3). In situ hybridization revealed that *Wnts2, 3, 7a, 7b, 10a* and *16* are expressed in the

epidermis (Fig. 3a, b, f, g, h); *Wnts5a* and *11* in the dermis (Fig. 3d, j); *Wnts4, 6* and *10b* in both layers (Fig. 3c-e, i, k). Furthermore, the *Wnts3, 4* and *6* transcripts were evenly distributed throughout the epidermis, including the hair placode and interfollicular epithelium (Fig. 3b-c, e). While *Wnts2, 7b, 10a* and *10b* showed elevated expression in the hair placode (Fig. 3a, g-i), *Wnts7a* and *16* were expressed mainly in the interfollicular epithelium (Fig. 3f, k). The inactivation of *Gpr177* abolished the epidermal expression of *Wnts2, 7a, 7b, 10a* and *10b* (Fig. 3a', f', g'-i'). In contrast, *Wnts3, 4, 6* and *16* genes remained active in the $Gpr177^{K5}$ mutants (Fig. 3b'-c', e', k'). Furthermore, the dermal expression of *Wnt5a and Wnt11* genes was affected by the epidermal ablation of Gpr177 (Fig. 3d' and j'). Their expression at low uniform levels was maintained in the dermis but absent in the dermal condensate due to the lack of hair placodes in the mutants. Based on these data, we could categorize the hair follicle-expressing *Wnt* genes into two groups: the first one, consisting of *Wnts3, 4* and *6*, was evenly expressed in the hair placode and interfollicular epithelium and the second one, consisting of *Wnts2, 7b, 10a* and *10b*, exhibited elevated expression in the hair placode. The second, but not the first, group was affected by the epidermal deletion of Gpr177. The results imply a potential hierarchy of Wnt activation during hair follicle development.

Because β-catenin signaling is required for early onset of hair follicle morphogenesis (Huelsken et al., 2001; Zhang et al., 2009), we examined if the canonical Wnt pathway was affected by the Gpr177 deletion. Immunostaining of the control and $Gpr177^{K5}$ skins revealed a drastic reduction of an activated form of β-catenin (ABC) in the hair placode and the underlying mesenchyme at E14.5 (Fig. 3l, l'), and in the hair follicular epithelial and dermal cells at E17.5 (Fig. 3m, m'). The reduction of active β-catenin was also accompanied by the loss of expression of its transcriptional targets, Axin2 (Fig. 3n-o, n'-o'), Lef1 (Fig. 3p, p') and Dkk4 (Fig. 3q, q'), all of which are critically involved in hair follicle development (Bazzi et al., 2007; van Genderen et al., 1994; Yu et al., 2005a). An early activation of Wnt/β-catenin signaling in the upper dermis immediately adjacent to the epidermis occurs prior to the appearance of the placode at E13.5 (Fig. 3n). This early activation is totally disrupted by the epidermal deletion of Gpr177 (Fig. 3n'), suggesting that Wnt secretion from the epidermis drives the early response of β-catenin signaling in the dermis. Subsequently, the patterned signaling activity of Wnt/β-catenin necessary for hair follicle morphogenesis is impaired (Huelsken et al., 2001; Zhang et al., 2009). In addition to cell fate specification and differentiation, Wnt is also a key signal for cell proliferation and survival during skin organogenesis (Widelitz, 2008). BrdU incorporation analysis revealed that the number of proliferating cells was significantly reduced in the epidermis, but not dermis, of $Gpr177^{K5}$, compared to the littermate control (Fig. S4a-c, mutant: 23.91±0.01% and control: 34.03±0.03%; p value <0.01, n=3). In contrast, no alteration in programmed cell death was detected (Fig. S4d-i). These results indicated a detrimental effect of the Gpr177 deletion on the canonical Wnt pathway, suggesting a mechanism underlying the induction of hair follicles mediated through Gpr177-dependent β-catenin signaling.

To definitively assess that the impairment of β-catenin signaling is responsible for the hair follicle defects, we introduced the β-catΔEx3Fx allele into the $Gpr177^{K5}$ mice to generate the $Gpr177^{K5}$; $s\beta cat^{K5}$ model. In these mutants, a stabilized β-catenin mutant was expressed

in the developing epidermis due to the deletion of exon 3 of β-catenin (Maruyama et al., 2010; Mirando et al., 2010). By restoring the signaling activity of β-catenin in the epidermal cells, we examined if the block in hair follicle induction caused by the Gpr177 deletion could be alleviated. Hair placode-like structures were apparent in the $Gpr177^{K5}$; $s\beta cat^{K5}$ skins, suggesting that induction has occurred at E15.5 (Fig. 4a-c; 100%, n=3). Immunostaining analysis further showed that epidermal activation of β-catenin takes place in the absence of Gpr177 (Fig. 4d-i). Nonetheless, dermal activation of β-catenin remained absent in the $Gpr177^{K5}$; $s\beta cat^{K5}$ skins (Fig. 4g-i). To demonstrate the proper induction of hair follicles, we first examined the expression of Edar and Shh, which are evident in the control but missing in the $Gpr177^{K5}$ skins (Fig. 4j-k, m-n). However, expression of the β-catenin mutant was able to activate Edar and Shh in the placode-like structures of $Gpr177^{K5}$; $s\beta cat^{K5}$, suggesting a restoration of placode signals (Fig. 4l, o) and follicular epithelium positive for K17 (Fig. 4p-r). Therefore, β-catenin activation was able to overcome the block in hair follicle induction caused by the epidermal deletion of Gpr177. The formation of hair placodes then triggers a clustering of underlying mesenchymal cells to form dermal condensates, leading to the development of dermal papilla expressing CD133 (Hardy, 1992; Ito et al., 2007; Millar, 2002). The CD133 positive dermal papilla cells were present in the control, but absent in the $Gpr177^{K5}$; $s\beta cat^{K5}$ mutants (Fig. 4s-u). Analysis of another dermal papilla marker, Sox2, showed similar results (Fig. 4v-x). Although epidermal activation of β-catenin alleviated the defects associated with initial induction of hair follicles, it failed to induce the neighboring mesenchymal cells to form dermal condensates. These data indicate that Wnt/β-catenin signaling induced by the epidermal Wnt is essential for initial induction of hair follicles within the epidermis. They also imply that the subsequent development of dermal papilla requires Wnt secretion from the epithelial cells to induce Wnt signaling in the mesenchymal cells.

To investigate the requirement of Wnt production contributing to the first dermal signal essential for hair follicle induction, we generated the $Gpr177^{Dermo1}$ model where Gpr177 was inactivated in the mesenchymal cells of the developing dermis by Dermo1-Cre (Tran et al., 2010). Using R26R, β-gal staining of the E14.5 skins showed the efficiency of Cre recombination in the mesenchymal cells of developing dermis (Fig. 5a-b). Immunostaining of Gpr177 further indicated its removal in the dermis, but not epidermis, of $Gpr177^{Dermo1}$ (Fig. 5c-f). The dermal deletion of Gpr177 had no effect on the formation of hair placodes at E15.5, suggesting that Gpr177-mediated Wnt production may be dispensable in the dermis for hair follicle initiation (Fig. 5g-h; 100%, n=8). Immunostaining of Cadherin proteins showed that the morphology of follicular epithelial cells in the mutants is comparable to that of control (Fig. 5i-l). Although Edar and Shh were expressed in the hair placodes, their expression was affected by the mutation (Fig. 5m-p). Quantitative measurement confirmed their expression domains reduced in the mutants (Fig. 5y). Nonetheless, there were sufficient placode signals to promote the subsequent development in the underlying mesenchyme as the dermal condensate formation was not affected in the $Gpr177^{Dermo1}$ mutants (Fig. 5q-r). Although the dermal deletion of Gpr177 had minimal effects on hair follicle induction, the dermal layer of $Gpr177^{Dermo1}$ exhibited a loose structure with significant abnormalities (Fig. S5). The number of proliferating cells was significantly reduced in the dermis, but not the epidermis, of $Gpr177^{Dermo1}$, compared to the littermate controls (Fig. S5a-c, mutant:

19.01±0.03% and control: 32.21±0.03%; *p* value <0.01, n=3). In contrast, no alteration in apoptosis was detected (Fig. S5d-i). The dermal deletion affected cell-type specification of mesenchymal cells into fibroblasts, smooth muscle cells and adipocytes modulated by Wnt (Wei et al., 2011). We detected an apparent shift of dermal cell identity to favor adipocytes in the $Gpr177^{Dermo1}$ mutants (Fig. S5j-p). This is consistent with prior reports indicating the differential effects of Wnt on development of the mesenchymal derived cell types (Cristancho and Lazar, 2011; Wei et al., 2011).

Analysis of ABC and Axin2 expression further indicated no significant difference in the pattern of Wnt/β-catenin signaling (Fig. 5s-y). In the upper dermis of $Gpr177^{Dermo1}$, the early activation of Wnt/β-catenin signaling remained detectable prior to the appearance of hair placodes at E13.5 (Fig. 5u-v) and was unaffected in the placode and dermal condensate at E14.5 (Fig. 5w-x). To ensure that Gpr177 is required for Wnt production in the dermis, the Wnt secretion assay was performed. While the control dermal cells were capable of activating the TOPFLASH reporter, this activation was reduced by the Gpr177 deletion, suggesting an impairment of Wnt secretion (Fig. S6a). Furthermore, activation of several Wnt signaling effectors was not affected in the dermis and epidermis of $Gpr177^{Dermo1}$ most likely due to the presence of epidermal Wnt (Fig. S6b). However, using the ex vivo culture of primary cells, we found that Wnt signaling activation is impaired in the $Gpr177^{Dermo1}$ dermal cells (Fig. S6b). Although Gpr177 regulates Wnt secretion in the dermis, dermal Wnt is not required for hair follicle induction. Dermal Wnt seems to be dispensable for the early events of hair follicle formation. The findings thus do not support the idea that Wnt is secreted from the dermis to induce β-catenin signaling in the epidermis essential for hair follicle initiation.

Discussion

This study reveals that epidermal Gpr177 is essential for the Wnt-mediated induction of hair follicles. Epidermal, but not dermal, deletion of Gpr177 disrupts the early activation of Wnt/β-catenin signaling in the upper dermis prior to initiation of the hair follicle. Subsequently, the expression of Edar, Bmp2/4 and Shh, critical for hair follicle morphogenesis is disrupted (Botchkarev and Sharov, 2004; Laurikkala et al., 2002; St-Jacques et al., 1998), suggesting that Wnt signaling acts upstream of these pathways. These results are consistent with recent findings but provide additional insights into hair follicle induction mediated by Wnt signaling crosstalk between the epidermis and dermis. Our data also support the requirement of Gpr177 for secretion of all canonical Wnts, and imply a hierarchy of Wnt actions during hair follicle formation. Furthermore, the lack of hair placode formation upon deletion of Gpr177 in the epidermis can be alleviated by β-catenin stimulation, suggesting that epidermal autocrine signaling of Wnt promotes the induction process. However, epidermal restoration of β-catenin signaling fails to correct the subsequent formation of dermal condensates. Due to the lack of epidermal Wnt, the dermal papilla does not develop properly. Epidermal Wnt is therefore a part of the placode signals which induce the dermis through an extra-epidermal signaling mechanism. Our Dermo1-mediated deletion of Gpr177 causes defects in dermal cell proliferation and differentiation, as well as in full induction of the placode signal during hair follicle morphogenesis, not detected in the deletion mediated by En1-Cre (Chen et al., 2012). Although Wnt secretion is

impaired, β-catenin signaling remains intact in both epidermis and dermis of the mutants. The induction of hair follicle and dermal papilla is mainly unaffected. These surprising results argue against Wnt being the initial dermal signal.

We propose a mechanism underlying hair follicle development mediated by the Wnt pathway based on previous evidence and current findings (Fig. 5z). β-catenin signaling is first uniformly activated in the upper dermis prior to its activation in the epithelium of hair placodes or feather buds (Noramly et al., 1999; Zhang et al., 2009). This is followed by activation in the placode epithelium, and then in the underlying mesenchyme and dermal condensate (DasGupta and Fuchs, 1999; Fuchs, 2007). Epidermal but not dermal production of Wnt is responsible for activating β-catenin signaling in the epithelium and mesenchyme during hair follicle and dermal papilla induction. Epidermal Wnt is required for activation of β-catenin signaling not only within the epidermis (epidermal autocrine) but also across the epidermis to the dermis (epidermal paracrine). Before the receipt of the initial dermal signal, β-catenin signaling is blocked by an inhibitor (χ) in the epidermis (Stage: Pre-placode). However, the epidermal Wnt signal permits a uniform activation of β-catenin signaling in the upper dermis via epidermal paracrine regulation. When the initial dermal signal arises, it prohibits the inhibitory effect on β-catenin to induce the placode-specific signaling in the epidermis, followed by induction of the hair placode via epidermal autocrine regulation (Stage: Placode). A reciprocal induction occurs subsequently between the placode and underlying mesenchyme (Stage: Dermal condensate). The placode signals including epidermal Wnt are sent back to the underlying mesenchyme to pattern the signaling activity of β-catenin for the formation of dermal condensate and papilla. Dermal Wnt as a feedback then promotes full induction of the placode signals.

The concept of the “first dermal message” as the initial signal for hair follicle induction was described decades ago (Hardy, 1992). Because of the essential role of β-catenin and its spatiotemporal expression pattern during hair follicle development, Wnt has been suggested to be the first signal in the dermis (Fuchs, 2007; Millar, 2002; Noramly et al., 1999; Schmidt-Ullrich and Paus, 2005). However, the identity of the initial dermal signal remains unknown. Our genetic studies imply that Wnt probably is not the “first dermal message”. Instead of activation, we hypothesize that the initial dermal signal is required for de-repression of β-catenin signaling in the epidermis. Smad7-mediated regulation of Smurf2 has been shown to modulate β-catenin in hair follicle formation (Han et al., 2006), suggesting a potential mechanism underlying the inhibition effect of Wnt. How the first signal arising in the dermis to regulate the Wnt pathway in the epidermis remains an important question to be addressed.

The dermal abnormalities detected in the $Gpr177^{Dermo1}$ mutants suggest that the Gpr177-mediated secretion of Wnt play a critical role in the dermis. These abnormalities are most likely caused by alteration in noncanonical, but not canonical, Wnt because the β-catenin signaling activity is comparable in the dermis of control and $Gpr177^{Dermo1}$. Furthermore, dermal Wnt is not required for hair follicle initiation, consistent with Wnt5a dispensable for this process (Hu et al., 2010).

Another question remaining to be addressed is which of the Wnt family members regulates the development of hair follicles. Our findings suggest that the epidermal deletion of Gpr177 interferes with the expression of *Wnts2, 7b, 10a* and *10b*, which exhibit elevated expression in the placode. However, the expression of *Wnts3, 4* and *6* uniformly in the interfollicular epithelium and placode is not affected in the mutants. As a trafficking regulator for Wnt proteins, the removal of Gpr177 does not usually affect their expression (Fu et al., 2011; Fu et al., 2009). Therefore, there is a hierarchy of Wnts controlling hair follicle development. The first group of *Wnt* genes, including *Wnts3, 4* and *6*, is unaffected by the Gpr177 deletion in the epidermis. Thus they represent candidates for the "primary Wnt" which mediates hair follicle initiation. The second group of *Wnt* genes, including *Wnts2, 7b, 10a* and *10b*, whose expression is disrupted by the Gpr177 deletion, depends on epidermal activation of β-catenin signaling. This is supported by the observation that ectopic expression of the Wnt inhibitor Dkk1 and ectodermal deletion of β-catenin impair hair follicle development through disruption of patterning signaling mediated by *Wnts 10a* and *10b* (Andl et al., 2002; Zhang et al., 2009). Therefore, *Wnts 2, 7b, 10a* and *10b* likely act as the "secondary Wnt", which is a part of the placode signal, essential for hair follicle development. Defining the specific role of these Wnts in hair follicle induction promises important insights into the mechanism underlying hair follicle morphogenesis.

Methods

Mouse strains and Cells

The Gpr177Fx, K5-Cre, Dermo1-Cre, R26R, β-catΔEx3Fx and Axin2^{lacZ} mouse strains, and genotyping methods were reported previously (Fu et al., 2011; Harada et al., 1999; Soriano, 1999; Sosic et al., 2003; Tarutani et al., 1997; Yu et al., 2005a; Yu et al., 2003). Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester. Epidermal and dermal cells isolated from the specific skin layer were cultured in the CnT-PCT and DMEM media, respectively, with 10% FBS. For Wnt secretion assay, cells transfected with TOPFLASH were used as indicator for receiving of Wnt signals determined by relative luciferase activity.

Histology, β-gal staining, immunostaining, immunoblot and TUNEL analysis

For histology and immunostaining, samples were fixed and embedded for sections, stained with H&E and specific antibodies using the avidin:biotinlylated based method (Chiu et al., 2008; Fu et al., 2011; Fu et al., 2009; Maruyama et al., 2010; Yu et al., 2005a; Yu et al., 2010; Yu et al., 2005b). β-gal staining was performed with standard protocols described previously (Fu et al., 2009; Maruyama et al., 2010; Yu et al., 2005b). To detect apoptotic cells, TUNEL staining was performed as described (Maruyama et al., 2010; Yu et al., 2007).

RT-PCR and In situ hybridization

Total RNA isolated from E14.5 mouse skins was subject to RT-PCR analysis. In situ hybridization was performed to detect gene expression using the digoxygenin-labeled probes, followed by recognition with an alkaline phosphatase conjugated anti-digoxygenin antibody (Chiu et al., 2008; David and Wedlich, 2001; Fu et al., 2009; Yu et al., 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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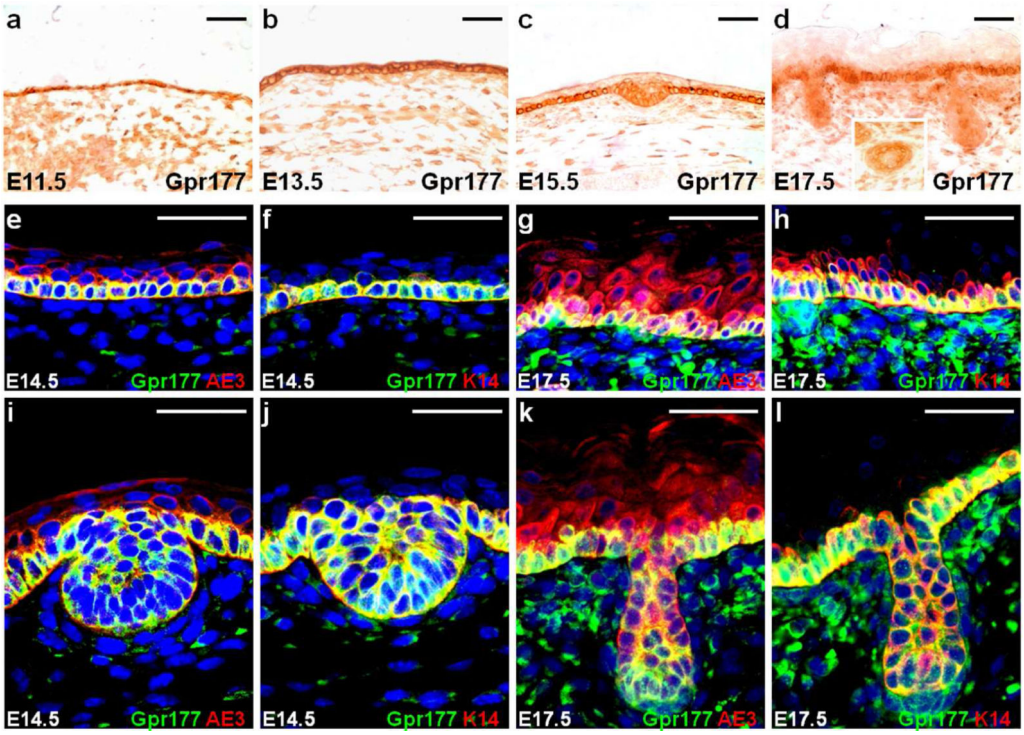

Fig. 1.

Gpr177 is expressed in hair follicle development. Immunostaining of the E11.5 (a) and E13.5 (b) skins shows the expression of Gpr177 in the epithelium and underlying mesenchyme. A restricted elevation is found in the epidermal basal cells and hair follicular cells at E15.5 (c) and E17.5 (d), respectively. The inset shows non-uniform expression of Gpr177 in the hair follicle (d). Double labeling of Gpr177 (e-l) with AE3, a marker for the entire epidermis (e, g, i, k), or K14, a marker for the epidermal basal layer (f, h, j, l), identifies the Gpr177-expressing cells at E14.5 (e-f, i-j) and E17.5 (g-h, k-l). Scale bars, 50 μm (a-l).

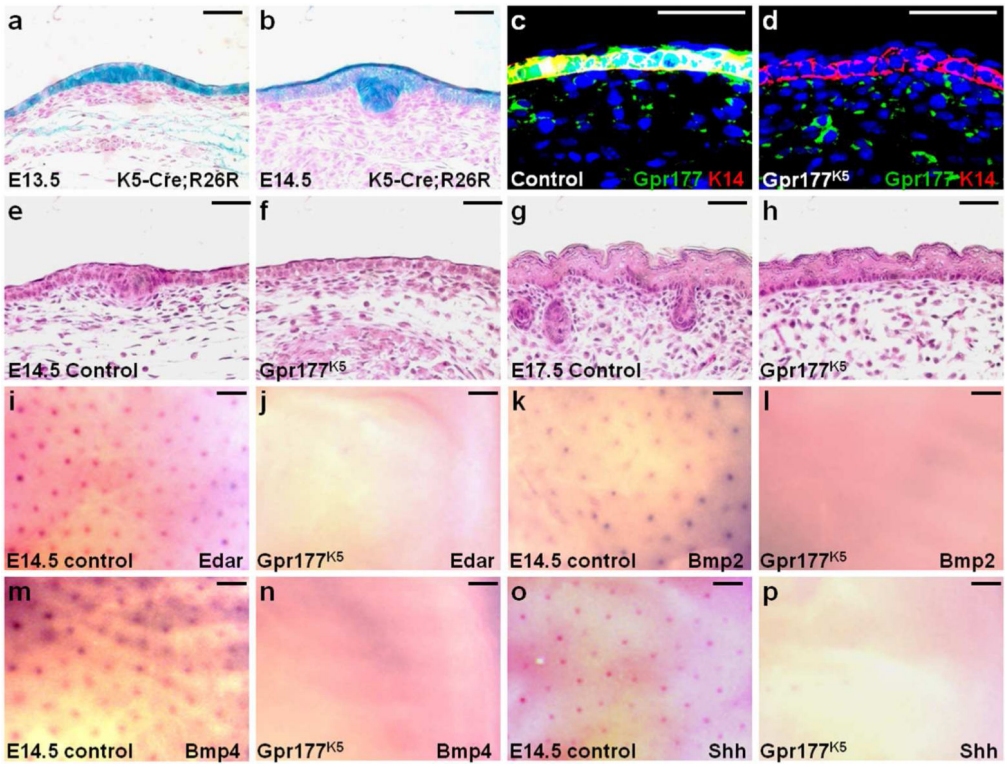

Fig. 2.

Epidermal deletion of Gpr177 abrogates the induction of hair follicles. β-gal staining of the E13.5 (a) and E14.5 (b) K5-Cre; R26R embryos analyzes the effectiveness of Cre recombination in the epidermis and hair placode. Sections of the control and Gpr177^{K5} embryos were analyzed by co-immunostaining of Gpr177 and K14 (c-d) and hematoxylin/eosin staining (e-h) at E14.5 (c-f) and E17.5 (g-h). Whole mount in situ hybridization of the control (i, k, m, o) and Gpr177^{K5} (j, l, n, p) embryos reveals the expression of Edar (i-j), Bmp2 (k-l), Bmp4 (m-n) and Shh (o-p) at E14.5. Control genotype: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+. Scale bars, 50 μm (a-h); 200 μm (i-p).

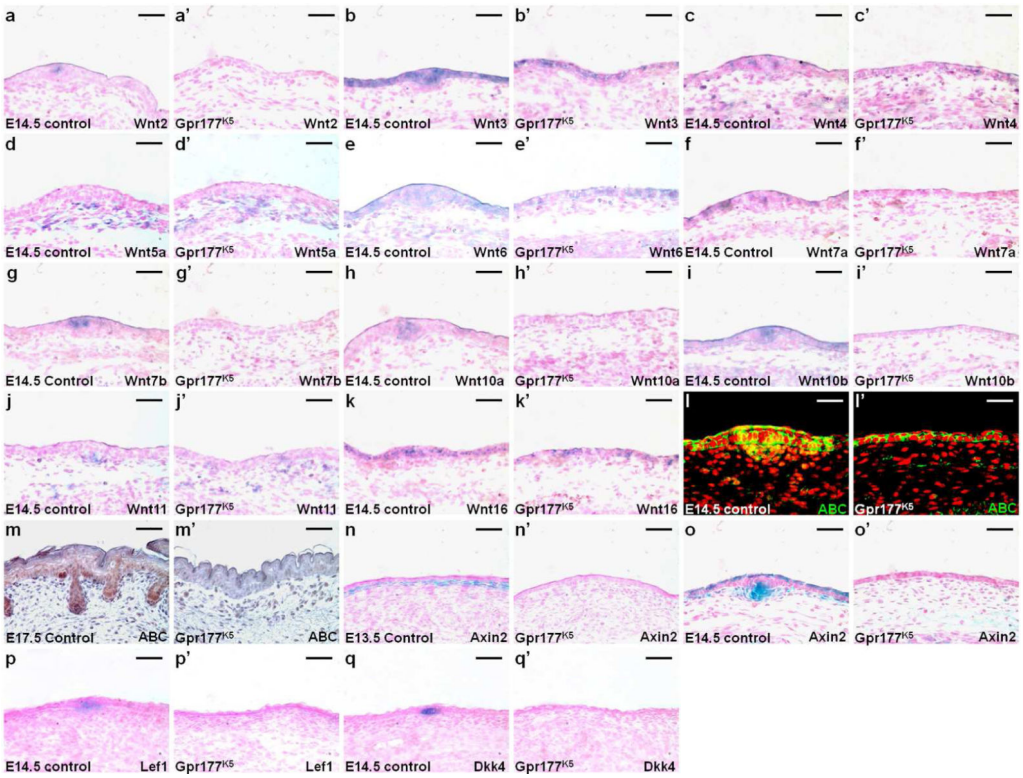

Fig. 3.

Wnt expression and signaling are affected by the epidermal deletion of Gpr177. In situ hybridization in sections shows the expression of *Wnt* genes in the E14.5 control (a-k) and $Gpr177^{K5}$ (a'-k') skins. Sections of control (l-o) and $Gpr177^{K5}$ (l'-o') skins examine the signaling activity of Wnt by immunostaining of an activated form of β-catenin (ABC) and β-gal staining of the $Axin2^{lacZ}$ allele at E13.5 (n, n'), E14.5 (l, l', o, o') and E17.5 (m, m'). In situ hybridization analyzes the expression of Wnt downstream targets, Lef1 (p, p') and Dkk4 (q, q') in the E14.5 control (p-q) and $Gpr177^{K5}$ (p'-q') skins. Control genotype: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+. Scale bars, 50 μm (a-q, a'-q').

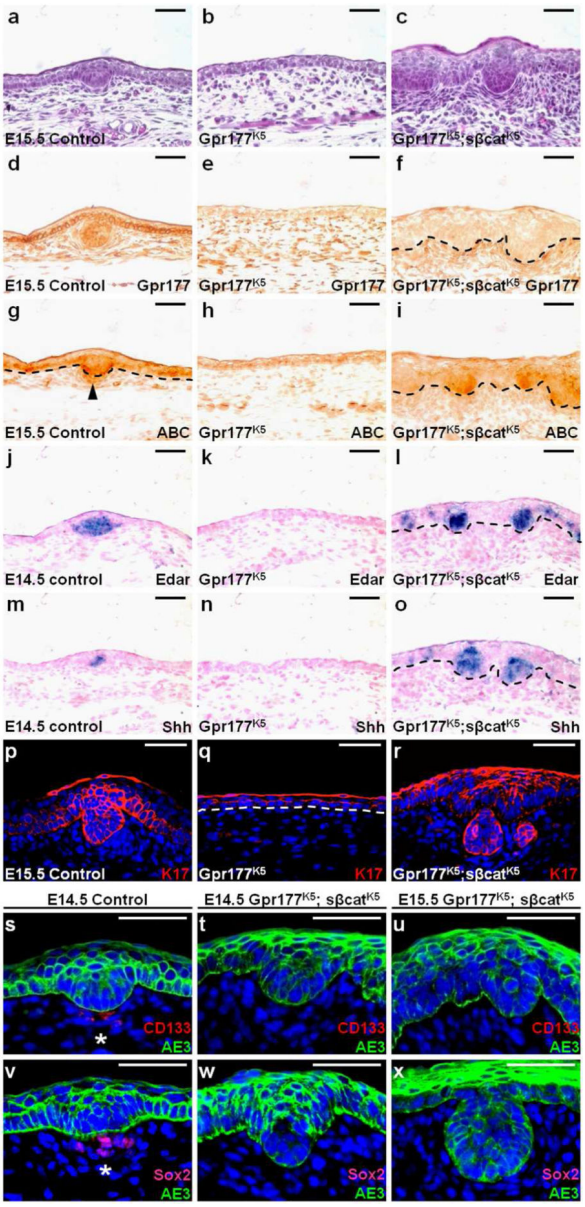

Fig. 4.

Epidermal stimulation of β-catenin alleviates the hair follicle defects of Gpr177^{K5}. Sections of the control, Gpr177^{K5} and Gpr177^{K5}; sβcat^{K5} were analyzed by H&E staining (a-c), immunostaining of Gpr177 (d-f), ABC (g-i) and K17 (p-r), in situ hybridization of Edar (j-l) and Shh (m-o), and double labeling of CD133 and AE3 (s-u) or Sox2 and AE3 (v-x) at E14.5 and E15.5. Arrowhead indicates the dermal activation of β-catenin in the control (g), but not in the Gpr177^{K5} and Gpr177^{K5}; sβcat^{K5} mutants (h-i). Asterisks indicate the dermal papilla markers, CD133 and Sox2, are detected in the control (s, v), but absent in the Gpr177^{K5}; sβcat^{K5} mutants (q-r, t-u). Control genotype: Gpr177Fx/Fx or K5-Cre. Scale bars, 50 μm (a-x).

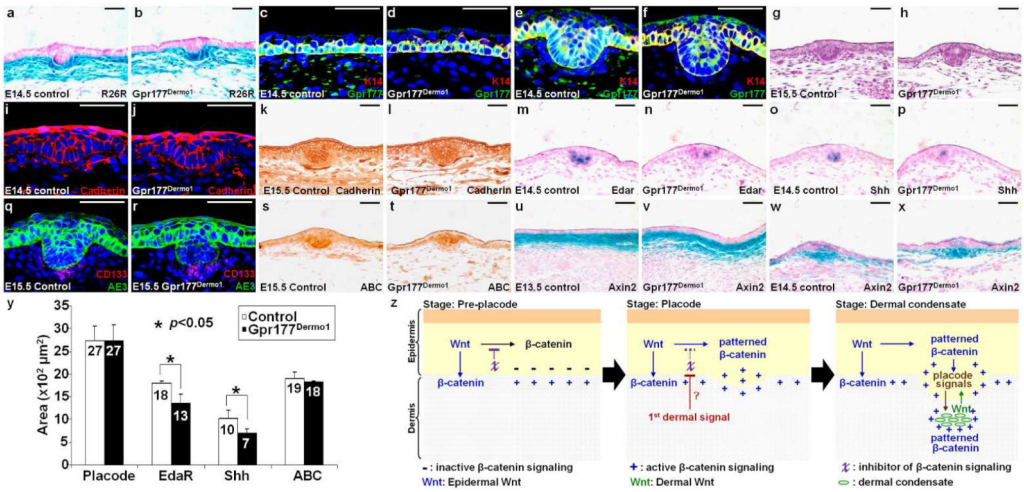

Fig. 5.

The Gpr177-mediated regulation of Wnt in the dermis is dispensable for hair follicle initiation. β-gal staining of the E14.5 control and Gpr177^{Dermo1} skins carrying R26R shows Cre effectiveness (a-b). Sections are analyzed by co-labeling of Gpr177 and K14 (c-f) or CD133 and AE3 (q-r), H&E (g-h), immunostaining of Cadherin (i-l) and ABC (s-t) and in situ hybridization of Edar (m-n) and Shh (o-p), and β-gal staining of the Axin2^{lacZ} allele (u-x) at E13.5, E14.5 and E15.5. (y) Graph indicates quantitative analysis for the placode region and the expression domain for ABC, Edar and Shh. Control genotype: Gpr177Fx/Fx or Dermo1-Cre; Gpr177Fx/+. Scale bars, 50 μm (a-x). (z) Model for epidermal Wnt in orchestrating signaling interaction between the epidermis and dermis during hair follicle development.