Epidermal Wnt Controls Hair Follicle Induction by Orchestrating Dynamic Signaling Crosstalk between the Epidermis and Dermis

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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* ortholog, is essential for hair follicle induction. *Gpr177*, encoding a multipass transmembrane protein, regulates Wnt sorting and secretion. Cell type–specific abrogation of the signal reveals that only epidermal, but not dermal, production of Wnt is required. An intraepidermal Wnt signal is necessary and sufficient for hair follicle initiation. However, 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.

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INTRODUCTION

The mammalian skin and its appendages are derived from ectoderm and mesoderm during embryogenesis (Hardy, 1992; Fuchs, 2007). The embryo surface emerges as a single layer of epithelial cells that give rise to the epidermis. The dermis is formed from the underlying mesoderm composed of mesenchymal cells. Subsequently, the epidermal–dermal interaction results in hair follicle formation (Hardy, 1992; Fuchs, 2007). 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 (Fuchs, 2007; Driskell *et al.*, 2011). 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 (Hardy, 1992; Millar, 2002; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007). 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). Thus, the data suggest the 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 the most critical regulator for early development of the epidermis (Noramly *et al.*, 1999; Millar, 2002; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007). Wnt blocks the ability of the ectoderm to respond to fibroblast growth factor. This in turn elevates bone morphogenic protein (BMP), leading to fate determination of the epidermis (Stern, 2005; Fuchs, 2007). 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 (DasGupta and Fuchs, 1999; Andl *et al.*, 2002; 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 (Gat *et al.*, 1998; Andl *et al.*, 2002). Although

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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, owing to the 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 ortholog of *Drosophila Wls/Evi/Srt* essential for proper sorting and secretion of Wnt (Banziger *et al.*, 2006; Bartscherer *et al.*, 2006; Goodman *et al.*, 2006; Fu *et al.*, 2009, 2011). In flies, *Wls/Evi/Srt* regulates the secretion of all Wnts, except for WntD, because of 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.*, 2009, 2011; 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 the 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 embryonic day 11.5 (E11.5) and E13.5 (Figure 1a and 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 (Figure 1c and d). Colabeling of Gpr177 with AE3, a marker for the entire epidermis, or keratin 14, a marker for the epidermal basal layer, further indicated that the epidermal basal cells and the hair follicular cells express high levels of Gpr177 (Figure 1e–l and Supplementary Figure S1 online).

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-galactosidase staining of the E13.5 and E14.5 skin showed the effectiveness of Cre recombination in the epidermis and developing hair follicles (Figure 2a and b). Immunostaining for Gpr177 further indicated its successful ablation in the epidermis, but not dermis, of Gpr177^{K5} (Figure 2c and d). Wnt secretion assays further examined the 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 cocultured with the signal-receiving cells harboring a TOPFLASH reporter for β-catenin and Lef/Tcf-dependent transcription. Although 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 (Supplementary Figure S2a online). Furthermore, the activation of several Wnt signaling mediators was affected in the epidermis of Gpr177^{K5} (Supplementary Figure S2b online). 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 (Figure 2e–h; 100%, $n=15$). Next, we examined the expression of placode/follicle-specific genes affected by the loss of Gpr177 (Laurikkala *et al.*, 2002; Botchkarev and Sharov, 2004; 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 (Figure 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 (Noramly *et al.*, 1999; Millar, 2002; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007).

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 (Millar *et al.*, 1999; Reddy *et al.*, 2001; Andl *et al.*, 2002). We first examined the expression of all 19 mouse *Wnt* genes in the E14.5 skin by reverse transcriptase–PCR analysis, and detected transcripts of *Wnts 2*, *3*, *4*, *5a*, *6*, *7a*, *7b*, *10a*, *10b*, *11*, and *16*, including those reported (Reddy *et al.*, 2001), whose expression patterns were further characterized (Supplementary Figure S3 online). *In situ* hybridization revealed that *Wnts 2*, *3*, *7a*, *7b*, *10a*, and *16* are expressed in the epidermis (Figure 3a, b, f, g, and h); *Wnts 5a* and *11* in the dermis (Figure 3d and j); and *Wnts 4*, *6*, and *10b* in both layers (Figure 3c–e, I, and k). Furthermore, the *Wnt 3*, *4*, and *6* transcripts were evenly distributed throughout the epidermis, including the hair placode and interfollicular epithelium (Figure 3b, c, and e). Although *Wnts 2*, *7b*, *10a*, and *10b* showed elevated expression in the hair placode (Figure 3a, g–i), *Wnts 7a* and *16* were expressed mainly in the interfollicular epithelium (Figure 3f and k). The inactivation of *Gpr177* abolished the epidermal expression of *Wnts 2*, *7a*, *7b*, *10a*, and *10b* (Figure 3a′, f′, g′–i′). In contrast, *Wnt 3*, *4*, *6*, and *16* genes remained active in the Gpr177^{K5} mutants (Figure 3b′, c′, e′, and k′). Furthermore, the dermal expression of *Wnt5a and Wnt11* genes was affected by the epidermal ablation of Gpr177 (Figure 3d′ and j′). Their expression at low uniform levels was maintained in the dermis but absent in the dermal condensate because of the lack of hair placodes in the mutants. On the basis of these data, we could categorize the hair follicle–expressing *Wnt* genes into two groups: the first one, consisting of *Wnts 3*, *4*, and *6*, was evenly expressed in the hair placode and interfollicular epithelium, and the second one, consisting of *Wnts 2*, *7b*, *10a*, and *10b*, exhibited

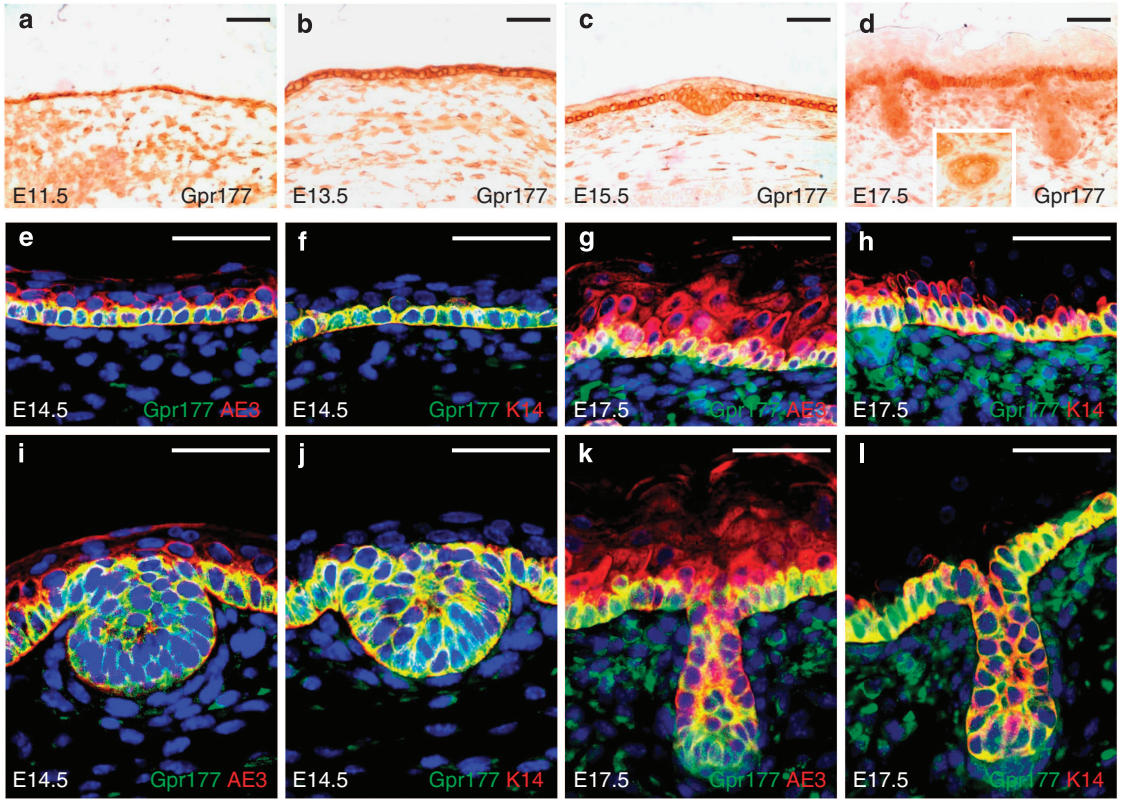

Figure 1. Gpr177 is expressed in hair follicle development. Immunostaining of the (**a**) embryonic day 11.5 (E11.5) and (**b**) E13.5 skin shows the expression of Gpr177 in the epithelium and underlying mesenchyme. A restricted elevation is found in the epidermal basal cells and hair follicular cells at (**c**) E15.5 and (**d**) E17.5, respectively. (**d**) The inset shows nonuniform expression of Gpr177 in the hair follicle. (**e**–**l**) Double labeling of Gpr177 with (**e**, **g**, **i**, **k**) AE3, a marker for the entire epidermis, or (**f**, **h**, **j**, **l**) keratin 14 (K14), a marker for the epidermal basal layer, identifies the Gpr177-expressing cells at (**e**, **f**, **i**, **j**) E14.5 and (**g**, **h**, **k**, **l**) E17.5. Bars = 50 μm (**a**–**l**).

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 whether the canonical Wnt pathway was affected by the Gpr177 deletion. Immunostaining of the control and Gpr177^{K5} skin revealed a drastic reduction of an activated form of β-catenin (ABC) in the hair placode and the underlying mesenchyme at E14.5 (Figure 3l and l′), and in the hair follicular epithelial and dermal cells at E17.5 (Figure 3m and m′). The reduction of active β-catenin was also accompanied by the loss of expression of its transcriptional targets, Axin2 (Figure 3n, o, n′ and o′), Lef1 (Figure 3p and p′), and Dkk4 (Figure 3q and q′), all of which are critically involved in hair follicle development (van Genderen *et al.*, 1994; Yu *et al.*, 2005a; Bazzi *et al.*, 2007). An early activation of Wnt/β-catenin signaling in the upper dermis immediately adjacent to the epidermis occurs before the appearance of the placode at E13.5 (Figure 3n). This early activation is totally disrupted by the epidermal deletion of Gpr177 (Figure 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 with the littermate control (Supplementary Figure S4a–c online, mutant: 23.91 ± 0.01% and control: 34.03 ± 0.03%; $P<0.01$, $n=3$). In contrast, no alteration in programmed cell death was detected (Supplementary Figure S4d–i online). 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}; sβcat^{K5} model. In these mutants, a stabilized β-catenin mutant was expressed in the developing epidermis because of 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 whether the block in hair follicle induction

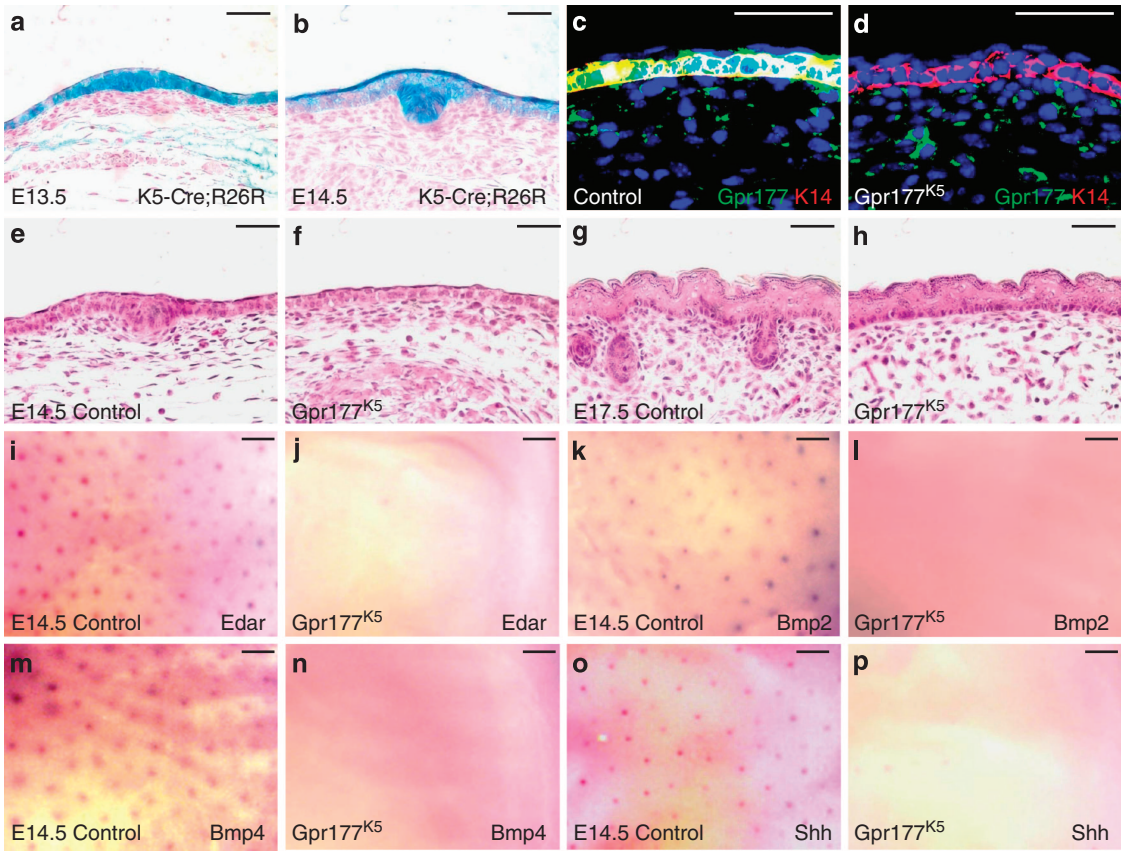

Figure 2. Epidermal deletion of Gpr177 abrogates the induction of hair follicles. β-Galactosidase (β-gal) staining of the (**a**) embryonic day 13.5 (E13.5) and (**b**) E14.5 K5-Cre; R26R embryos analyzes the effectiveness of Cre recombination in the epidermis and hair placode. Sections of the control and Gpr177^{K5} embryos were analyzed by coimmunostaining of (**c**, **d**) Gpr177 and keratin 14 (K14) and (**e**–**h**) hematoxylin/eosin staining at (**c**–**f**) E14.5 and (**g**, **h**) E17.5. Whole-mount *in situ* hybridization of the (**i**, **k**, **m**, **o**) control and (**j**, **l**, **n**, **p**) Gpr177^{K5} embryos reveals the expression of (**i**, **j**) Edar, (**k**, **l**) Bmp2, (**m**, **n**) Bmp4, and (**o**, **p**) Shh at E14.5. Control genotype: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+. Bars = 50 μm (**a**–**h**); 200 μm (**i**–**p**).

caused by the Gpr177 deletion could be alleviated. Hair placode–like structures were apparent in the Gpr177^{K5}; sβcat^{K5} skin, suggesting that induction has occurred at E15.5 (Figure 4a–c; 100%, $n = 3$). Immunostaining analysis further showed that epidermal activation of β-catenin takes place in the absence of Gpr177 (Figure 4d–i). Nonetheless, dermal activation of β-catenin remained absent in the Gpr177^{K5}; sβcat^{K5} skin (Figure 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} skin (Figure 4j, k, m, and n). However, expression of the β-catenin mutant was able to activate Edar and Shh in the placode-like structures of Gpr177^{K5}; sβcat^{K5}, suggesting a restoration of placode signals (Figure 4l and o) and follicular epithelium positive for keratin 17 (Figure 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; Millar, 2002; Ito *et al.*, 2007). The CD133-positive dermal papilla cells were present in the control, but absent in the Gpr177^{K5}; sβcat^{K5} mutants (Figure 4s–u). Analysis of another dermal papilla marker, Sox2, showed similar results (Figure 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-galactosidase staining of the E14.5 skin showed the efficiency of Cre recombination in the mesenchymal cells of developing dermis (Figure 5a and b). Immunostaining of Gpr177 further indicated its removal in the dermis, but not epidermis, of Gpr177^{Dermo1} (Figure 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

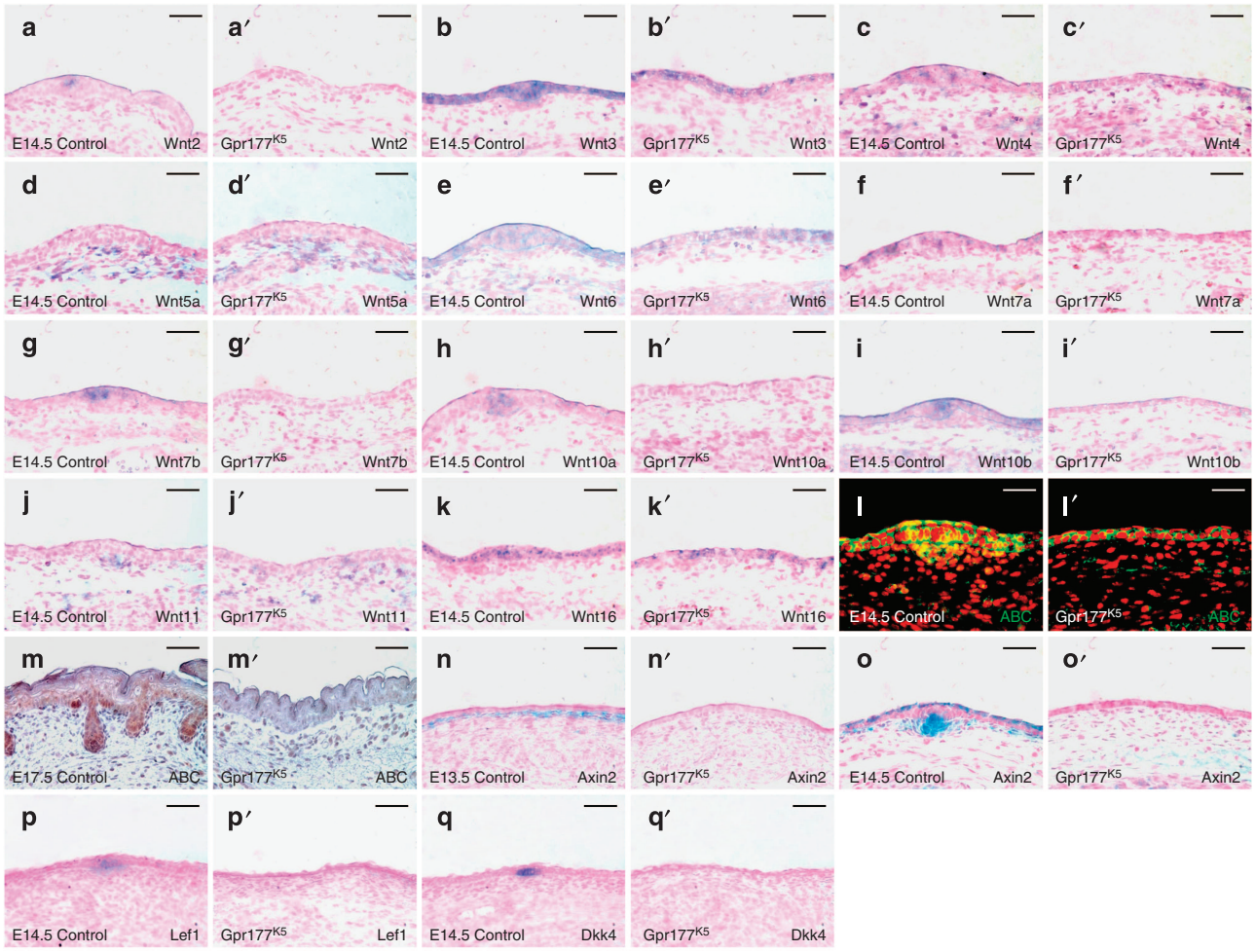

Figure 3. Wnt expression and signaling are affected by the epidermal deletion of Gpr177. *In situ* hybridization in sections shows the expression of *Wnt* genes in the (**a**–**k**) embryonic day 14.5 (E14.5) control and (**a′**–**k′**) Gpr177^{K5} skin. Sections of (**l**–**o**) control and (**l′**–**o′**) Gpr177^{K5} skin examine the signaling activity of Wnt by immunostaining of an activated form of β-catenin (ABC) and β-galactosidase (β-gal) staining of the Axin2^{lacZ} allele at (**n**, **n′**) E13.5, (**l**, **l′**, **o**, **o′**) E14.5, and (**m**, **m′**) E17.5. *In situ* hybridization analyzes the expression of Wnt downstream targets, (**p**, **p′**) Lef1 and (**q**, **q′**) Dkk4 in the E14.5 (**p**, **q**) control and (**p′**, **q′**) Gpr177^{K5} skin. Control genotype: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+. Bars = 50 μm (**a**–**q**, **a′**–**q′**).

dispensable in the dermis for hair follicle initiation (Figure 5g and 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 (Figure 5i–l). Although Edar and Shh were expressed in the hair placodes, their expression was affected by the mutation (Figure 5m–p). Quantitative measurement confirmed that their expression domains reduced in the mutants (Figure 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 (Figure 5q and 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 (Supplementary Figure S5 online). The number of proliferating cells was significantly reduced in the dermis, but not the epidermis, of Gpr177^{Dermo1} compared with the littermate controls (Supplementary Figure S5a–c online, mutant: 19.01 ± 0.03% and control: 32.21 ± 0.03%; $P < 0.01$, $n = 3$). In contrast, no alteration in apoptosis was detected (Supplementary Figure S5d–i online). 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 (Supplementary Figure S5j–p online). 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 (Figure 5s–y). In the upper dermis of Gpr177^{Dermo1}, the early activation of Wnt/β-catenin signaling remained detectable before the appearance of hair placodes at E13.5 (Figure 5u and v) and was unaffected in the placode and dermal

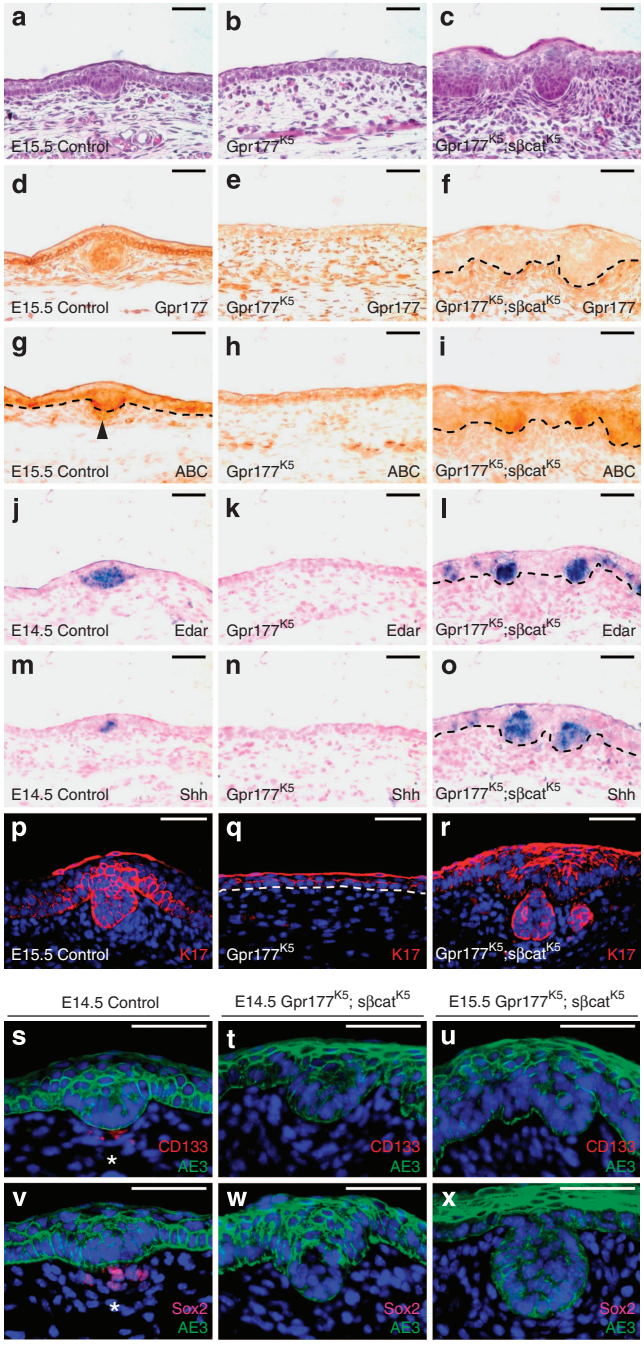

Figure 4. Epidermal stimulation of β-catenin alleviates the hair follicle defects of Gpr177^{K5}. Sections of the control, Gpr177^{K5}, and Gpr177^{K5}; sβcat^{K5} were analyzed by (**a**–**c**) hematoxylin and eosin (H&E) staining, (**d**–**f**) immunostaining of Gpr177, (**g**–**i**) ABC, and (**p**–**r**) keratin 17 (K17), *in situ* hybridization of (**j**–**l**) Edar and (**m**–**o**) Shh, and double labeling of (**s**–**u**) CD133 and AE3 or (**v**–**x**) Sox2 and AE3 at embryonic day 14.5 (E14.5) and E15.5. Arrowhead indicates the dermal activation of β-catenin in the (**g**) control, but not in the (**h**, **i**) Gpr177^{K5} and Gpr177^{K5}; sβcat^{K5} mutants. (**s**, **v**) Asterisks indicating the dermal papilla markers, CD133 and Sox2, are detected in the control, (**q**, **r**, **t**, **u**) but absent in the Gpr177^{K5}; sβcat^{K5} mutants. Control genotype: Gpr177Fx/Fx or K5-Cre. Bars = 50 μm (**a**–**x**).

condensate at E14.5 (Figure 5w and x). To ensure that Gpr177 is required for Wnt production in the dermis, the Wnt secretion assay was performed. Although 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 (Supplementary Figure S6a online). Furthermore, the activation of several Wnt signaling effectors was not affected in the dermis and epidermis of Gpr177^{Dermo1}, most likely because of the presence of epidermal Wnt (Supplementary Figure S6b online). However, using the *ex vivo* culture of primary cells, we found that the Wnt signaling activation is impaired in the Gpr177^{Dermo1} dermal cells (Supplementary Figure S6b online). 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. Thus, the findings 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 before the initiation of the hair follicle. Subsequently, the expression of Edar, Bmp2/4, and Shh, critical for hair follicle morphogenesis, is disrupted (St-Jacques *et al.*, 1998; Laurikkala *et al.*, 2002; Botchkarev and Sharov, 2004), 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 the 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. Owing to the lack of epidermal Wnt, the dermal papilla does not develop properly. Epidermal Wnt is therefore a part of the placode signals that induce the dermis through an extraepidermal 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 (Figure 5z). β-catenin signaling is first uniformly activated in the upper dermis before 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

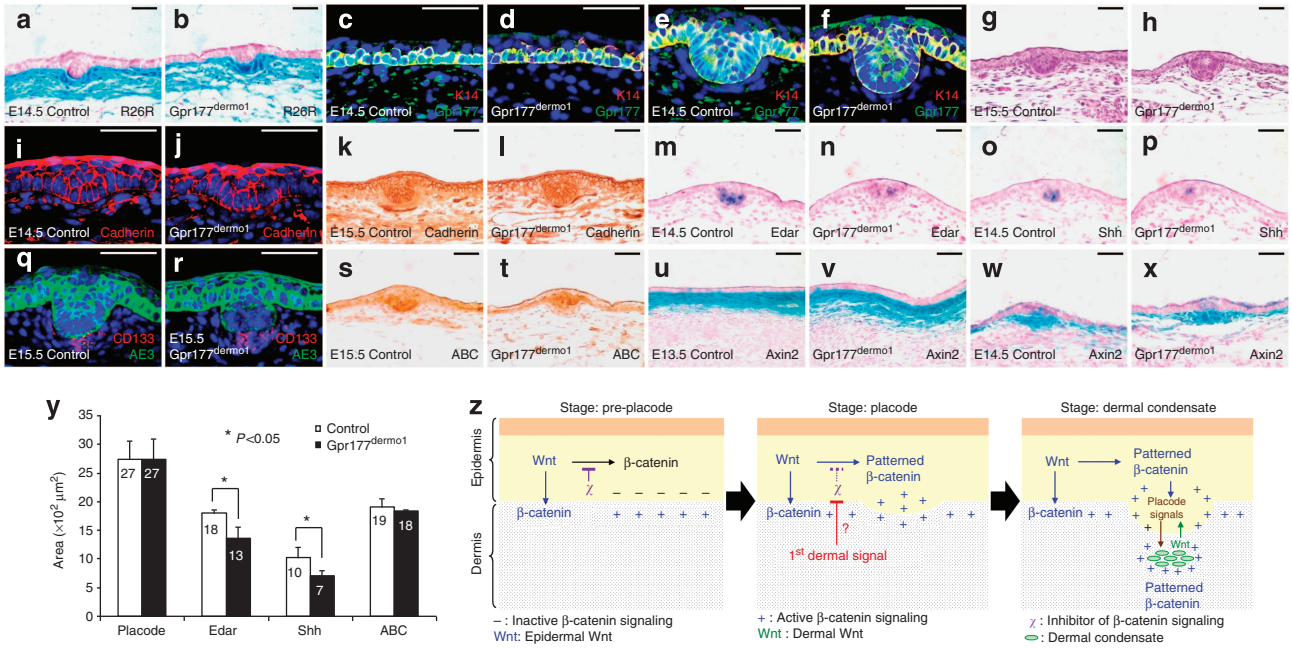

Figure 5. The Gpr177-mediated regulation of Wnt in the dermis is dispensable for hair follicle initiation. (**a**, **b**) β-Galactosidase (β-gal) staining of the embryonic day 14.5 (E14.5) control and Gpr177^{Dermo1} skin carrying R26R shows Cre effectiveness. Sections are analyzed by colabeling of (**c**–**f**) Gpr177 and keratin 14 (K14) or (**q**, **r**) CD133 and AE3, (**g**, **h**) hematoxylin and eosin (H&E), immunostaining of (**i**–**l**) Cadherin and (**s**, **t**) ABC and *in situ* hybridization of (**m**, **n**) Edar and (**o**, **p**) Shh, and (**u**–**x**) β-gal staining of the Axin2^{lacZ} allele 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/+. Bars = 50 μm (**a**–**x**). (**z**) Model for epidermal Wnt in orchestrating signaling interaction between the epidermis and dermis during hair follicle development.

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 (X) in the epidermis (stage: preplacode). However, the epidermal Wnt signal permits a uniform activation of β-catenin signaling in the upper dermis through 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 through 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 (Noramly *et al.*, 1999; Millar, 2002; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007). 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 derepression 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 arises 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 has 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 that remains to be addressed is which of the Wnt family members regulate the development of hair follicles. Our findings suggest that the epidermal deletion of Gpr177 interferes with the expression of *Wnts 2*, *7b*, *10a*, and *10b*, which exhibit elevated expression in the placode. However, the expression of *Wnts 3*, *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.*, 2009, 2011). Therefore, there is a hierarchy of Wnts controlling hair follicle development. The first group of *Wnt* genes, including *Wnts 3*, *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 *Wnts 2*, *7b*, *10a*, and *10b*, whose expression is disrupted by the Gpr177 deletion, depends on the 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.

MATERIALS AND 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 (Tarutani *et al.*, 1997; Harada *et al.*, 1999; Soriano, 1999; Sosic *et al.*, 2003; Yu *et al.*, 2003, 2005a; Fu *et al.*, 2011). 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% fetal bovine serum. For Wnt secretion assay, cells transfected with TOPFLASH were used as indicators for receiving of Wnt signals determined by relative luciferase activity.

Histology, β-galactosidasestaining, immunostaining, immunoblot, and TUNEL analysis

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

Reverse transcriptase–PCR and *in situ* hybridization

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

Experimental details are described in Supplementary Information online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Supplemental Information

Methods

Mouse strains

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). For generating Gpr177^{K5} mouse strain, mice carrying the K5-Cre transgene was first crossed with the Gpr177Fx/Fx mice to obtain the K5-Cre; Gpr177Fx/+ strain. The K5-Cre; Gpr177Fx/+ mice were then crossed with the Gpr177Fx/Fx mice to obtain mice carrying the Gpr177^{K5} (genotype: K5-Cre; Gpr177Fx/Fx). A similar breeding strategy was used to generate the Gpr177^{Dermo1} (genotype: Dermo1-Cre; Gpr177Fx/Fx) mutant strain. To examine the Cre activity, K5-Cre and Dermo1-Cre mice were bred into the R26R heterozygous background to obtain the K5-Cre; R26R and Dermo1-Cre; R26R mice, respectively. To simultaneously delete Gpr177 and monitor the Cre-mediated recombination, Gpr177^{Dermo1} mice were crossed into the R26R background to obtain the Gpr177^{Dermo1}; R26R mutants. The deletion of Gpr177 and expression of lacZ reporter occurred when Cre was expressed. For detecting the Axin2 expression, K5-Cre; Gpr177Fx/+ mice was crossed with mice homozygous for Gpr177Fx and Axin2^{lacZ} to create the Gpr177^{K5}; Axin2^{lacZ} (genotype: K5-Cre; Gpr177Fx/Fx; Axin2^{lacZ}+/-) mice. To express the stabilized β-catenin mutant protein in the Gpr177^{K5} mice, the K5-Cre; Gpr177Fx/+ mice were crossed with mice carrying Gpr177Fx/Fx and β-catΔEx3Fx/+ to generate the Gpr177^{K5}; sβcat^{K5} strain. 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.

Cells

To isolate epidermal and dermal cells, the dorsolateral skins were dissected from the E14.5 embryos. The skin tissues were then incubated in 0.5% Dispase (STEMCELL Technologies Inc., Vancouver, BC, Canada) at 4^{o}C for 2 hours. The epidermal and dermal layers were separated from each other, followed by dissociating into single cells. Primary epidermal and dermal cells were cultured in the CnT-PCT (CellnTEC, Switzerland) and DMEM media, respectively, with 10% FBS in a humidified chamber with 5% CO2 at 37^{o}C. For detection of Wnt secretion, 293T cells were transfected with TOPFLASH and RL-TK plasmids and co-cultured with the dermal cells for 24 hours. Relative luciferase activity was determined using a dual reporter luciferase kit (Promega, Madison, WI, USA).

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

Samples were fixed in formaldehyde or paraformaldehyde and then embedded to obtain paraffin sections which were stained with hematoxylin and eosin for histology or antibodies for immunological staining with avidin:biotinlylated enzyme complex as described (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). The immunological staining was visualized by enzymatic color reaction or fluorescence according to the manufacture's specification (Vector Laboratories, Burlingame, CA, USA). Images were taken using Zeiss Axio Observer microscope (Carl Zeiss, Thornwood, NY, USA). Immunoblot analysis was performed as described (Fu et al., 2009). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemical luminescence-mediated visualization (GE Healthcare Biosciences, Pittsburgh, USA) and autoradiography. Mouse monoclonal antibodies ABC (Millipore, Billerica, MA, USA), AE3 (Millipore), β-catenin (BD Biosciences, San Jose, CA, USA) BrdU (Thermo Scientific, Fremont, CA, USA), Collagen I (Abcam, Cambridge, MA, USA), Keratin 14

(Thermo Scientific) and SMA (Thermo Scientific); rabbit polyclonal antibodies Adiponectin (ProSci, Poway, CA, USA), CD133 (Novus, Littleton, CO, USA), Dvl2 (Cell Signaling Technology, Danvers, MA), Keratin 17 (Abcam), phosphor-GSK3 (Cell Signaling Technology) and Gpr177 (Fu et al., 2011; Fu et al., 2009; Yu et al., 2010); rabbit monoclonal antibody active caspase-3 (BD Biosciences) were used in these analyses. Details for β-gal staining in whole mounts or sections were described previously (Fu et al., 2009; Maruyama et al., 2010; Yu et al., 2005b). TUNEL staining was performed using ApopTag (Millipore) as described (Maruyama et al., 2010; Yu et al., 2007).

RT-PCR analysis

Total RNA isolated from E14.5 mouse skins was subject to the first strand cDNA synthesis using oligoT primers in 20 μl for 1 hour at 50^{o}C. The cDNA was then amplified by PCR (35 cycles, 94^{o}C for 15 seconds, 58^{o}C for 30 seconds and 72^{o}C for 60 seconds) in 50 μl buffered solution containing 1 μl of the diluted reverse transcription product in the presence of 20 pmoles each of the sense and antisense primers specific for the various target sequences as listed.

Wnt 1 Forward	ATGAACCTTCACAACAACGAG
Wnt 1 Reverse	GGTTGCTGCCTCGGTTG
Wnt 2 Forward	CTGGCTCTGGCTCCCTCTG
Wnt 2 Reverse	GGAACTGGTGTTGGCACTCTG
Wnt 2b Forward	CGTTCGTCTATGCTATCTCGTCAG
Wnt 2b Reverse	ACACCGTAATGGATGTTGTCACTAC
Wnt 3 Forward	CAAGCACAACAATGAAGCAGGC
Wnt 3 Reverse	TCGGGACTCACGGTGTTTCTC
Wnt 3a Forward	CACCACCGTCAGCAACAGCC
Wnt 3a Reverse	AGGAGCGTGTCACTGCGAAAG
Wnt 4 Forward	GAGAAGTGTGGCTGTGACCGG
Wnt 4 Reverse	ATGTTGTCCGAGCATCCTGACC
Wnt 5a Forward	CTCCTTCGCCCAGGTTGTTATAG
Wnt 5a Reverse	TGTCTTCGCACCTTCTCCAATG
Wnt 5b Forward	ATGCCCGAGAGCGTGAGAAG

Wnt 5b Reverse	ACATTTGCAGGCGACATCAGC
Wnt 6 Forward	TGCCCGAGGCGCAAGACTG
Wnt 6 Reverse	ATTGCAAACACGAAAGCTGTCTCTC
Wnt 7a Forward	CGACTGTGGCTGCGACAAG
Wnt 7a Reverse	CTTCATGTTCTCCTCCAGGATCTTC
Wnt 7b Forward	TCTCTGCTTTGGCGTCCTCTAC
Wnt 7b Reverse	GCCAGGCCAGGAATCTTGTTG
Wnt 8a Forward	ACGGTGGAATTGTCCTGAGCATG
Wnt 8a Reverse	GATGGCAGCAGAGCGGATGG
Wnt 8b Forward	TTGGGACCGTTGGAATTGCC
Wnt 8b Reverse	AGTCATCACAGCCACAGTTGTC
Wnt 9a Forward	GCAGCAAGTTTGTCAAGGAGTTCC
Wnt 9a Reverse	GCAGGAGCCAGACACACCATG
Wnt 9b Forward	AAGTACAGCACCAAGTTCCTCAGC
Wnt 9b Reverse	GAACAGCACAGGAGCCTGACAC
Wnt 10a Forward	CCTGTTCTTCCTACTGCTGCTGG
Wnt 10a Reverse	CGATCTGGATGCCCTGGATAGC
Wnt 10b Forward	TTCTCTCGGGATTTCTTGGATTC
Wnt 10b Reverse	TGCACTTCCGCTTCAGGTTTTC
Wnt 11 Forward	CTGAATCAGACGCAACACTGTAAAC
Wnt 11 Reverse	CTCTCTCCAGGTCAAGCAGGTAG
Wnt 16 Forward	AGTAGCGGCACCAAGGAGAC
Wnt 16 Reverse	GAAACTTTCTGCTGAACCACATGC
Shh Forward	GGAACTCACCCCCAATTACA
Shh Reverse	GAAGGTGAGGAAGTCGCTGT
Edar Forward	GCCCTACATGTCCTGTGGAT
Edar Reverse	GGCCTGAGAGCTCTTTGTGA
BMP2 Forward	AGGCGAAGAAAAGCAACAGA
BMP2 Reverse	GTCTCTGCTTCAGGCCAAAC
BMP4 Forward	TGAGAGACCCCAGCCTAAGA
BMP4 Reverse	AAACTTGCTGGAAAGGCTCA
Lef1 Forward	CACACATCCCGTCAGATGTC
Lef1 Reverse	TGAGGCTTCACGTGCATTAG
Dkk4 Forward	GTGGAAGACACAAGGCCAGT
Dkk4 Reverse	TGGAGCAGACTTGTCCCTCT

In situ hybridization

In situ hybridization analysis was performed as described (Chiu et al., 2008; Fu et al., 2009; Yu et al., 2010). In brief, embryos were incubated with digoxygenin labeled probes, followed by recognition with an alkaline phosphatase conjugated anti-digoxygenin antibody. To visualize the bound signals, samples were incubated with BM-purple for 4-5 hours. The RNA

probes were generated using a PCR based method described previously (David and Wedlich, 2001). Briefly, T3 or T7 promoter sequences were introduced to the 5-prime end of the reverse and forward primers, enabling the synthesis of antisense and sense transcripts, respectively. PCR fragments were then amplified using gene-specific primers and purified with Quick-spin columns (Qiagen Inc., Valencia, CA, USA), followed by generation of the digoxigenin labeled probes using T3 or T7 RNA polymerase. Quantification of the positively stained domains was performed by measuring areas with J-image software (n=3 individual experiments, mean ± SD).

Fig. S1. Gpr177 is expressed in hair follicle development. Double labeling of Gpr177 with AE3, a marker for the entire epidermis, or K14, a marker for the epidermal basal layer, identifies the Gpr177-expressing cells at E14.5 and E17.5. Sections are counterstained by DAPI. Panels show the individually stained and superimposed images. Scale bars, 50 μm.

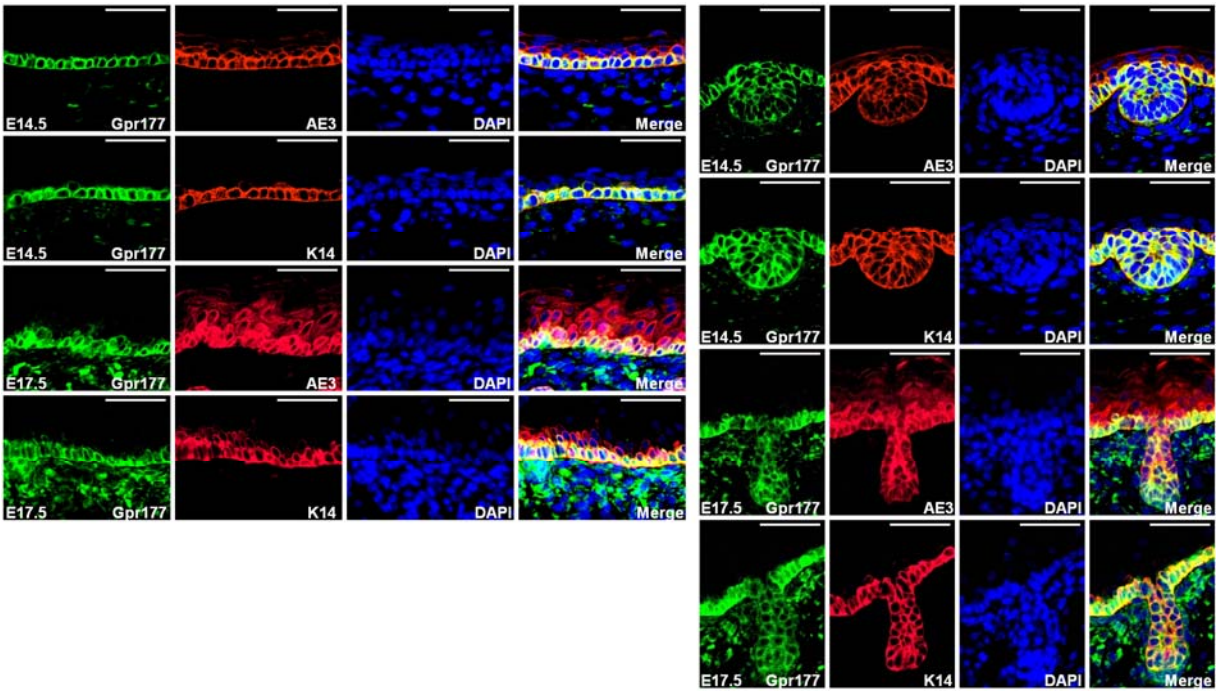

Fig. S2. Wnt production and signaling is impaired in the Gpr177^{K5} mutants. (a) Primary epidermal cells isolated from the E14.5 control and Gpr177^{K5} skins as the signal-producing cells were co-cultured with the signal-receiving cells harboring the TOPFLASH reporter. Relative luciferase activity (RLA) determined activation of the β-catenin and Lef/Tcf-dependent transcription. The addition of canonical Wnt3a and noncanonical Wnt5a in the media was used as the positive and negative controls, respectively. Asterisk indicates the statistical significance of reduction (*p* value <0.01, n=3). (b) Immunoblot analysis of Dvl2, phosphorylated Dvl2 (pDvl2), phosphorylated GSK3, activated β-catenin (ABC), β-catenin (β-cat) determined the canonical Wnt signaling activity in the E14.5 epidermis of control and Gpr177^{K5}. The level of pDvl2, pGSK3 and ABC is elevated upon stimulation of Wnt signaling (Logan and Nusse, Annu Rev Cell Dev Biol, 2004). The epidermal deletion of Gpr177 reduces activation of Dvl2, GSK3 and β-catenin in the epidermis. Actin level is used as a loading control.

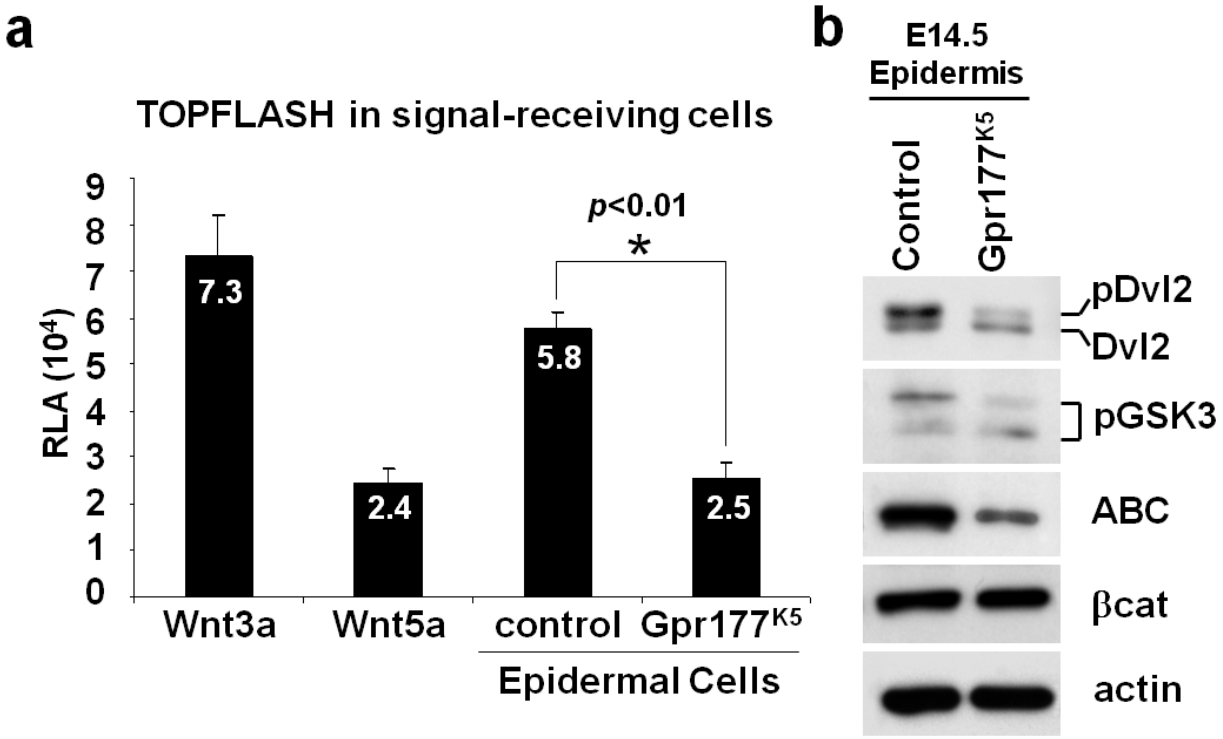

Fig. S3. Multiple members of the Wnt family are expressed in developing embryonic skin. RT-PCR analysis detects the transcript of *Wnts 2*, *3*, *4*, *5a*, *6*, *7a*, *7b*, *10a*, *10b*, *11* and *16*, but not *Wnts 1*, *2b*, *3a*, *5b*, *8a*, *8b*, *9a* and *9b* in the E14.5 skins.

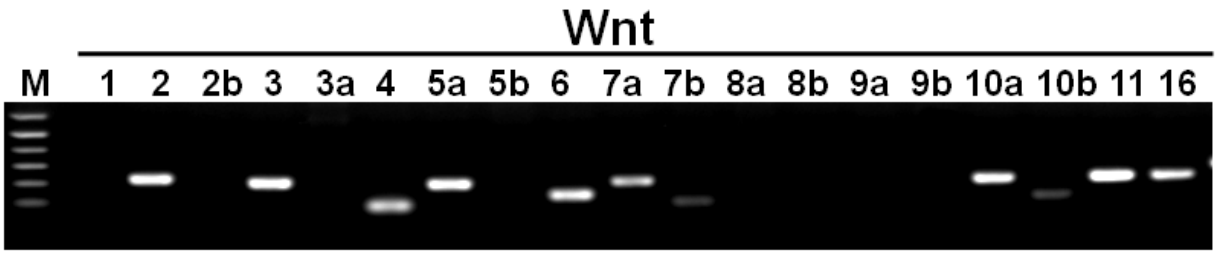

Fig. S4. Epidermal cell proliferation is affected by Gpr177 deficiency. Using BrdU incorporation assay, cells undergoing mitotic division are identified by immunostaining of BrdU in the E14.5 control (a) and Gpr177^{K5} (b) skins. Graph shows the percentage of proliferating cells positive for BrdU in the epidermis and dermis (c). Asterisk indicates that the reduction in the epidermis caused by the epidermal deletion of Gpr177 is statistically significant (Gpr177^{K5}: 23.91±0.01% and control: 34.03±0.03%; *p* value <0.01, n=3). TUNEL staining (d-f) and immunostaining of activated caspase3 (g-i) detect apoptotic cells in the skins (d, e, g, h) and dorsal root ganglia (DRG; f, i) of control (d, f, g, i) and Gpr177^{K5} (e, h) at E14.5. Broken lines indicate the epidermal-dermal junction and the DRG. Genotype – Control: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+ and Gpr177^{K5}: K5-Cre; Gpr177Fx/Fx. Scale bars, 50 μm (a, b, d-i).

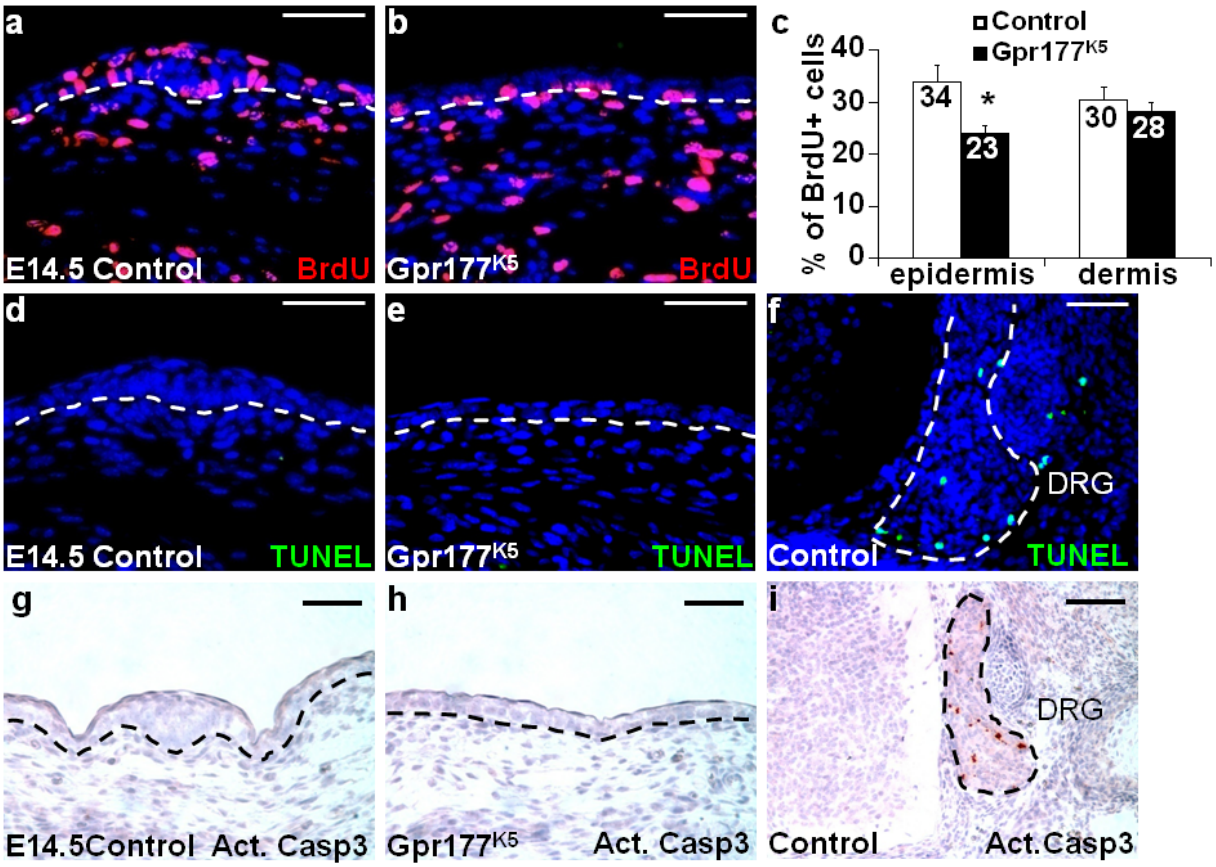

Fig. S5. Deletion of Gpr177 in the dermis causes dermal abnormalities. Using BrdU incorporation assay, cells undergoing mitotic division are identified by immunostaining of BrdU in the E14.5 control (a) and $Gpr177^{Dermo1}$ (b) skins. Graph shows the percentage of proliferating cells positive for BrdU in the epidermis and dermis (c). Asterisk indicates that the reduction in the dermis caused by the dermal deletion of Gpr177 is statistically significant ($Gpr177^{Dermo1}$: 19.01±0.03% and control: 32.21±0.03%; *p* value <0.01, n=3). TUNEL staining (d-f) and immunostaining of activated caspase3 (g-i) detect apoptotic cells in the skins (d, e, g, h) and DRG (f, i) of control (d, f, g, i) and $Gpr177^{Dermo1}$ (e, h) skins at E14.5. Sections of the E15.5 control (j, l, n) and $Gpr177^{Dermo1}$ (k, m, o) skins are analyzed by immunostaining of Collagen I (Col 1; j, k), smooth muscle actin (SMA; l, m) and adiponectin (AdipoNT; n, o). Graph shows the percentage of cells positive for the differentiation markers in the dermis (p). Asterisks indicates that the alterations of differentiation caused by the dermal deletion of Gpr177 are statistically significant (*, *p* value <0.01; **, *p* value <0.05; n=3). Broken lines indicate the epidermal-dermal junction and the DRG. Genotype – Control: Gpr177Fx/Fx or Dermo1-Cre; Gpr177Fx/+ and $Gpr177^{Dermo1}$: Dermo1-Cre; Gpr177Fx/Fx. Scale bars, 50 μm (a, b, d-o).

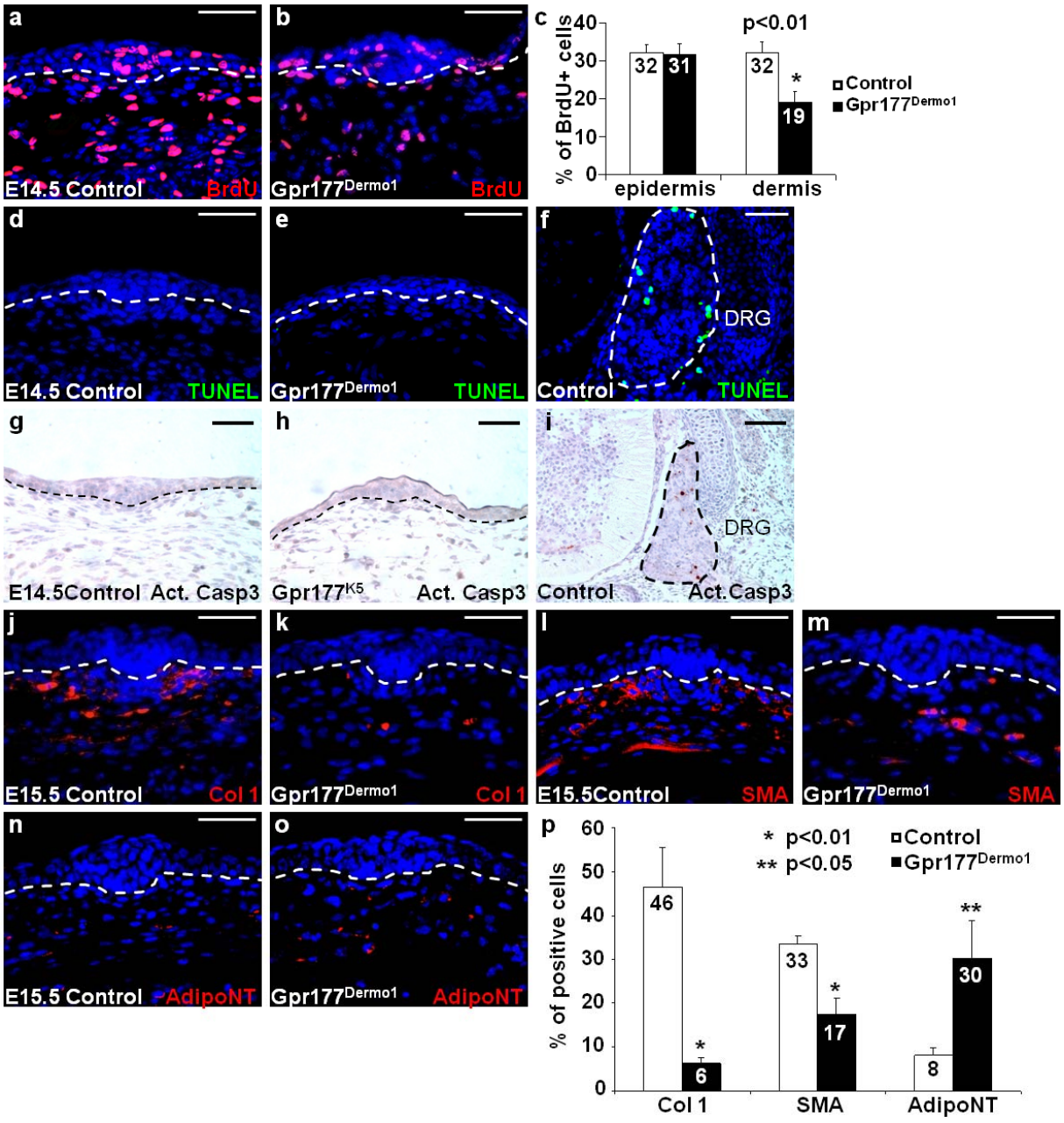
a
E14.5 Control
BrdU
b
Gpr177Dermo1
BrdU
c
p<0.01
% of BrdU+ cells
40
30
20
10
0
32
31
32
*
19
epidermis
dermis
Control
Gpr177Dermo1
d
E14.5 Control
TUNEL
e
Gpr177Dermo1
TUNEL
f
DRG
Control
TUNEL
g
E14.5Control
Act. Casp3
h
Gpr177K5
Act. Casp3
i
DRG
Control
Act.Casp3
j
E15.5 Control
Col 1
k
Gpr177Dermo1
Col 1
l
E15.5Control
SMA
m
Gpr177Dermo1
SMA
n
E15.5 Control
AdipoNT
o
Gpr177Dermo1
AdipoNT
p
* p<0.01
** p<0.05
Control
Gpr177Dermo1
% of positive cells
60
50
40
30
20
10
0
46
*
6
33
*
17
8
**
30
Col 1
SMA
AdipoNT

Fig. S6. Wnt secretion is impaired in the Gpr177^{Dermo1} mutants. (a) Primary dermal cells isolated from the E14.5 control and Gpr177^{Dermo1} skins as the signal-producing cells were co-cultured with the signal-receiving cells harboring the TOPFLASH reporter. Relative luciferase activity (RLA) determined activation of the β-catenin and Lef/Tcf-dependent transcription. The addition of canonical Wnt3a and noncanonical Wnt5a in the media was used as the positive and negative controls, respectively. Asterisk indicates the statistical significance of reduction (*p* value <0.013, n=3). (b) Immunoblot analysis of Dvl2, phosphorylated Dvl2 (pDvl2), phosphorylated GSK3, activated β-catenin (ABC), β-catenin (β-cat) determined the canonical Wnt signaling activity in the E14.5 epidermis and dermis, and the primary dermal cells of control and Gpr177^{Dermo1} in vivo and ex vivo, respectively. The level of pDvl2, pGSK3 and ABC is elevated upon stimulation of Wnt signaling. The dermal deletion of Gpr177 causes either a slight reduction or no obvious effects on activation of Dvl2, GSK3 and β-catenin most likely due to the presence of epidermal Wnt in vivo. However, activation of canonical Wnt signaling was significantly reduced in the Gpr177^{Dermo1} dermal cells free of epidermal cells in the ex vivo culture. Actin level is used as a loading control.