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Characterization of *Wnt* gene expression in developing and postnatal hair follicles and identification of *Wnt5a* as a target of Sonic hedgehog in hair follicle morphogenesis

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Abstract

Mutations in WNT effector genes perturb hair follicle morphogenesis, suggesting key roles for WNT proteins in this process. We show that expression of *Wnts 10b* and *10a* is upregulated in placodes at the onset of follicle morphogenesis and in postnatal hair follicles beginning a new cycle of hair growth. The expression of additional *Wnt* genes is observed in follicles at later stages of differentiation. Among these, we find that *Wnt5a* is expressed in the developing dermal condensate of wild type but not Sonic hedgehog (*Shh*)-null embryos, indicating that *Wnt5a* is a target of SHH in hair follicle morphogenesis. These results identify candidates for several key follicular signals and suggest that WNT and SHH signaling pathways interact to regulate hair follicle morphogenesis.

Keywords: Hair follicle; *Wnt*; Sonic hedgehog; Mouse; Skin; Signaling; Epidermis; Dermis; Dermal condensate; Dermal papilla; Outer root sheath; Inner root sheath; Gene expression; Morphogenesis; Basal cell carcinoma; Placode; Hair shaft; Anagen; Telogen; Catagen; Hair growth cycle

1. Introduction

Because of its accessibility and ability to regenerate, the hair follicle provides a uniquely useful model system for studying mechanisms of intercellular signaling and the interactions between different signaling pathways in development. Inappropriate activation of signaling pathways that operate in hair follicles causes several common skin tumors including basal cell carcinoma (BCC) and pilomatricoma, a tumor of hair follicle matrix cells (Chan et al., 1999; Chiang et al., 1999; Gat et al., 1998; Hahn et al., 1996; Johnson et al., 1996; Oro et al., 1997; St-Jacques et al., 1998; Unden et al., 1996). Identification of the signaling molecules and pathways directing hair follicle morphogenesis and the postnatal hair growth cycle also therefore forms an important component in our understanding of pathogenic states of the skin and may ultimately permit the development of novel therapies for skin tumors as well as for hair loss disease.

The initiation of hair follicle development requires a series of reciprocal inductive interactions between the epithelium and mesenchyme (Hardy, 1992) (Fig. 1A). The first signal directing hair follicle formation arises in the mesenchyme and causes the overlying epidermis to thicken, forming a placode (Hardy, 1992). Placode formation involves a shape change in the epithelial cells, which become more elongated than adjacent non-placode cells. Signals from the placode cause the clustering of underlying dermal cells to form a dermal condensate and a 'second dermal signal' from the condensate regulates the proliferation and downward movement of epithelial cells into the dermis, forming a hair germ (Hardy, 1992). As hair follicle development continues, epithelial cells surround the dermal condensate, which develops into the hair follicle dermal papilla. The epithelial cells differentiate to form concentric layers that are easily distinguishable histologically, and include the medulla, cortex and cuticle of the hair shaft and the three layers of the inner root sheath (Sperling, 1991) (Fig. 1B). This process is likely to require lateral communication between cells in different epithelial layers, as well as signaling between the epithelial and dermal

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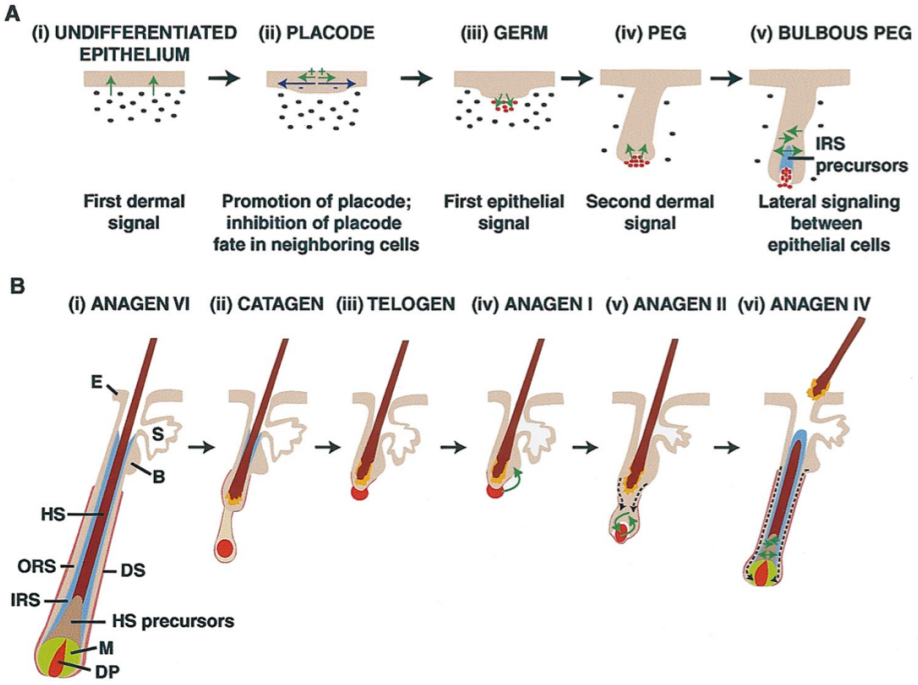

Fig. 1. Hair follicle morphogenesis and the hair growth cycle. (A) Schematic diagram of hair follicle morphogenesis. (i) The initial signal directing hair follicle formation arises in the mesenchyme and instructs the overlying epithelium to thicken, forming a placode. (ii) Placode formation is facilitated by promoting signals (+), shown in green, and prevented in neighboring epithelial cells by inhibitory signals (−), shown in blue. (iii) Signals from the epithelium induce the clustering of mesenchymal cells to form a dermal condensate (red cells). (iv) The dermal condensate signals to the follicular epithelium to proliferate and grow down into the dermis. (v) The dermal condensate becomes enveloped by follicular epithelial cells to form the dermal papilla. Differentiation of the inner root sheath may be regulated in part by lateral signaling between epithelial cells. (B) Events of the hair growth cycle. (i) During anagen, matrix cells derived from stem cells in the bulge proliferate and differentiate to form the inner root sheath and hair shaft. (ii) Anagen is followed by a period of regression, catagen, when the lower two-thirds of the follicle undergo programmed cell death. (iii) When catagen is completed, the follicle enters a resting phase, telogen. (iv) At the onset of a new cycle of hair growth, follicular epithelial stem cells are stimulated to divide by signals from the dermal papilla (green arrow). (v) During anagen signals from the dermal papilla stimulate the division of matrix cells and the inductive properties of the dermal papilla are maintained by signals from the follicular epithelium (green arrows). Progenitor cells originating in the bulge move down the outer root sheath to populate the hair follicle bulb (dashed black arrows), where they contribute to the matrix. (vi) Differentiation of matrix cells into hair shaft and inner root sheath is likely to involve lateral signaling between epithelial cells (green arrows). IRS, inner root sheath; E, epidermis; HS, hair shaft; ORS, outer root sheath; DP, dermal papilla; M, matrix; DS, dermal sheath; B, bulge; S, sebaceous gland.

In postnatal animals, hair follicles undergo cycles of growth (anagen), regression (catagen) and rest (telogen) that continue throughout life (Dry, 1926) (Fig. 1B). At the onset of a new cycle of hair growth, signals from the dermal papilla are thought to cause the transient proliferation of epithelial stem cells residing in the hair follicle bulge and the movement of their progeny to the lower part of the follicle (Cotsarelis et al., 1990; Oliver and Jahoda, 1988; Oshima et al., 2001; Taylor et al., 2000; Wilson et al., 1994). Subsequent proliferation and differentiation of stem cell progeny (matrix cells) result in the formation of a new hair shaft and inner root sheath (Oshima et al., 2001; Taylor et al., 2000). Movement of progenitor cells from the bulge to the matrix has been demonstrated to continue throughout anagen in vibrissa follicles (Oshima et al., 2001). The events of anagen bear striking similarities to those of hair follicle induction and development and like these processes require

Members of the fibroblast growth factor (FGF) and bone morphogenic protein (BMP) families of intercellular signaling molecules, together with ectodysplasin (EDA), transforming growth factor β2 (TGF-β2), noggin and Delta-1, have been implicated in hair follicle induction (Barsh, 1999; Botchkarev et al., 1999; Foitzik et al., 1999; Millar, 1997; Oro and Scott, 1998). The signaling molecule Sonic hedgehog (SHH) is dispensable for follicle induction, but is required for later stages of follicular development (Chiang et al., 1999; Karlsson et al., 1999; St-Jacques et al., 1998). TGF-β2 is also required for later morphogenetic stages to proceed normally (Foitzik et al., 1999). However, several key signals required for hair follicle formation remain partially characterized or unidentified. These include the ‘first dermal signal’ from mesenchyme to epithelium that causes induction of the hair follicle placode; the ‘first epithelial signal’ from epithelium to mesenchyme that initi-

signal' from dermal condensate to epithelium, which causes proliferation and differentiation of follicular epithelial cells (Hardy, 1992). Similarly, while SHH is known to be required for progression of anagen (Wang et al., 2000), little is known about the intercellular signaling molecules regulating anagen onset. Signaling molecules required for hair shaft and inner root sheath differentiation are likely to include Notch and BMPs (Kulessa et al., 2000; Lin et al., 2000; Powell et al., 1998), but are incompletely characterized and the signals controlling movements of progenitor cells from the bulge to the lower part of the follicle at anagen onset and during anagen are entirely unknown.

Wnt genes encode short-range secreted signaling molecules that regulate cell fate, adhesion, shape, proliferation, differentiation and movement, and are required for the development of multiple organ systems (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). WNT proteins can be grouped into two functional classes. Class I WNTs act through a 'canonical' signaling pathway that requires Dishevelled (DVL) protein and causes stabilization of cytoplasmic β-catenin and its translocation to the nucleus, where it forms transcriptional complexes with members of the lymphoid enhancer factor/T cell factor (LEF/TCF) family of DNA binding factors to control the expression of WNT target genes (Wodarz and Nusse, 1998). Class II WNTs operate via less well-characterized pathways that mediate proliferation, cell polarity and cell movements in gastrulation (Heisenberg et al., 2000; Miller et al., 1999; Sheldahl et al., 1999; Slusarski et al., 1997). Regulation of cell polarity and movements requires DVL, but not β-catenin (Axelrod et al., 1998; Djiane et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000).

While the properties of WNT proteins make them excellent candidates as regulators of hair follicle development and hair growth, several specific observations make this hypothesis compelling. Firstly, a loss of function mutation in mouse *Lef1* causes absence of vibrissae and greatly reduced numbers of body hair follicles (van Genderen et al., 1994), whereas over-expression of *Lef1* in the epidermis results in misangled and ectopic hair follicles (Zhou et al., 1995). Secondly, nuclear β-catenin is detected in both the epidermal and mesenchymal components of developing feather follicles (Noramly et al., 1999). Ablation of the β-catenin gene in mouse epidermis causes failure of hair follicle placode formation (Huelsken et al., 2001), while expression of stabilized forms of β-catenin in the epidermis results in the formation of ectopic, misangled feathers in chick embryos and additional hair follicles and hair follicle tumors in postnatal transgenic mice (Gat et al., 1998; Noramly et al., 1999; Widelitz et al., 2000). Stabilizing mutations in β-catenin have also been implicated as causative agents in human pilomatricoma, a tumor of hair follicle matrix cells (Chan et al., 1999). Further evidence for roles for β-catenin and LEF/TCF factors in developing and cycling hair follicles was provided by a promoter whose activity is induced by β-catenin/LEF and β-catenin/TCF complexes (DasGupta and Fuchs, 1999). Activity of the reporter gene was detected in the epithelium and mesenchyme of hair follicles at early stages of their formation, in hair shaft precursor cells during anagen, and in the bulge, the location of follicular epithelial stem cells (Cotsarelis et al., 1990; Morris and Potten, 1999; Taylor et al., 2000), at anagen onset. A role for WNT signaling in hair shaft formation is supported by the observations that *Wnt3* and DVL2 are expressed in hair shaft precursor cells and that ectopic expression of *Wnt3* in the outer root sheath disrupts hair shaft differentiation (Millar et al., 1999). In mice in which β-catenin is progressively lost from the epidermis and follicular epithelium, the first postnatal anagen phase is not initiated, providing functional evidence that WNT signals are required for anagen onset (Huelsken et al., 2001). Lastly, freshly isolated dermal papilla cells are capable of inducing epithelial cells to form a new hair follicle, but lose this property when maintained in culture. In the presence of Class I WNT proteins, the inductive abilities of cultured dermal papilla cells are retained, suggesting that, in vivo, WNT signals from the follicular epithelium act to maintain the function of the dermal papilla (Kishimoto et al., 2000).

Taken together, these observations suggest that WNT signals play key roles in hair follicle morphogenesis, hair shaft differentiation and follicular cycling. Despite several reports of *Wnt* gene expression in feather follicles (Christiansen et al., 1995; Chuong et al., 1996; Noramly et al., 1999; Tanda et al., 1995; Widelitz et al., 1999) and hair follicles (Kishimoto et al., 2000; St-Jacques et al., 1998; Millar et al., 1999), a systematic analysis of *Wnt* expression in developing and mature hair follicles to identify specific WNT proteins that are candidates for these signals has been lacking. We have carried out a comprehensive survey of *Wnt* gene expression in embryonic and postnatal skin and have identified three *Wnt* genes, *Wnts 10a*, *10b* and *5a*, whose expression is specifically upregulated in hair follicles at early morphogenetic stages. Of these, *Wnt10b* shows dramatic upregulation at the earliest stage of hair follicle development and is also specifically expressed in postnatal hair follicles at the onset of a new cycle of hair growth. Additional *Wnt* genes are expressed in specific subsets of cells in mature anagen follicles. We find that *Wnt5a* requires SHH for its expression in developing hair follicles, suggesting it as a target of SHH signaling. This observation has important implications for the study of BCC, a common skin tumor that shows similarity to immature hair follicles and is caused by inappropriate activation of the SHH signaling pathway.

2. Results

2.1. Multiple Wnt genes are expressed in embryonic skin

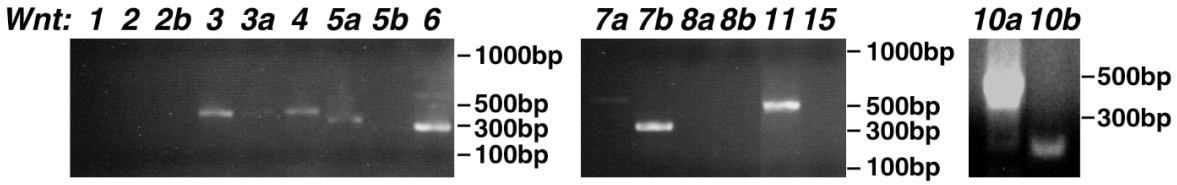

Fig. 2. RT-PCR analysis indicates that multiple members of the *Wnt* gene family are expressed in embryonic mouse skin. RNA was extracted from the dorsal skin of single mouse embryos at E14.5 and cDNA was synthesized by reverse transcription. *Wnt* cDNA fragments were amplified using specific primers. PCR products of the expected sizes were amplified using primers for *Wnts 3* (412 bp), *4* (431 bp), *5a* (349 bp), *6* (298 bp), *7b* (331 bp), *10a* (355 bp), *10b* (168 bp) and *11* (492 bp); faint bands of the expected sizes were detected for *Wnts 3a* (421 bp) and *7a* (550 bp); products of the expected sizes were not detected for *Wnts 1* (400 bp), *2* (496 bp), *2b* (454 bp), *5b* (291 bp), *8a* (410 bp), *8b* (453 bp), or *15* (299 bp) in this experiment.

we carried out reverse transcription-polymerase chain reaction (RT-PCR) experiments using specific primers for all currently identified mouse *Wnt* genes and RNA isolated from skin dissected from embryos at embryonic day 14.5 (E14.5). We designed the *Wnt* primers to span introns so that cDNA products could be distinguished from the products of amplification of any contaminating genomic DNA. In addition, control experiments in which the reverse transcriptase enzyme was omitted were performed for each primer pair and RNA preparation. Before experiments were carried out on embryonic skin RNA, each primer pair was tested on RNA prepared from whole embryos to ensure that a product of the appropriate size could be amplified. Expression of *Wnts 3*, *4*, *5a*, *6*, *7b*, *10a*, *10b* and *11* (Adamson et al., 1994; Christiansen et al., 1995; Gavin et al., 1990; Roelink et al., 1990; Wang and Shackleford, 1996) was detected in these experiments. In addition, weak signals were detected for *Wnts 3a* and *7a* (Gavin et al., 1990; Roelink et al., 1990). Expression of *Wnts 1*, *2*, *2b*, *5b*, *8a*, *8b* and *15* (Bergstein et al., 1997; Bouillet et al., 1996; Gavin et al., 1990; Nusse and Varmus, 1982; Richardson et al., 1999) was not detected in E14.5 skin (Fig. 2).

To determine the cellular localization of expression for *Wnt* genes positive for expression at E14.5 by RT-PCR and to determine whether *Wnts 1, 2*, *3a*, *7a*, *5b*, *8a*, *8b* or *15* are expressed in the skin or hair follicles at later developmental stages, probes for these genes were used for in situ hybridization experiments with sectioned embryos at E14.5, E15.5, E16.5 and E18.5. A probe for *Wnt16*, a recently cloned mouse *Wnt* gene (McWhirter et al., 1999) was also used for in situ hybridization experiments. Hybridization to internal embryonic organs provided a positive control for each probe. Sense probes were used as negative controls. *Wnts 3*, *4*, *6*, *7a*, *7b*, *10a*, *10b* and *16* are expressed in epidermal cells throughout embryogenesis (Fig. 3A,B,D–H,J). Probes for *Wnts 3*, *4*, *6*, *7b*, *10a* and *10b* give moderate to high signals and *Wnts 7a* and *16* produce very weak signals in the epidermis. In particular, the signal for *Wnt7a* is barely above background in the epidermis, although the probe hybridizes strongly to internal structures (Fig. 3E and data not shown). *Wnts 5a* and *11* are expressed at low levels in the dermis throughout embryogenesis (Fig. 3C,I). uniformly in the placode and interfollicular epithelium at E14.5 (Fig. 3A) but at later developmental stages is downregulated in follicular epithelium (data not shown), whereas *Wnts 4*, *6* and *7b* are weakly expressed in follicular epithelium throughout embryogenesis (Fig. 3B,D,F and data not shown). The specific localization of *Wnt7a* transcripts to developing follicles was not observed at any stage (Fig. 3E and data not shown). This result is in contrast to the

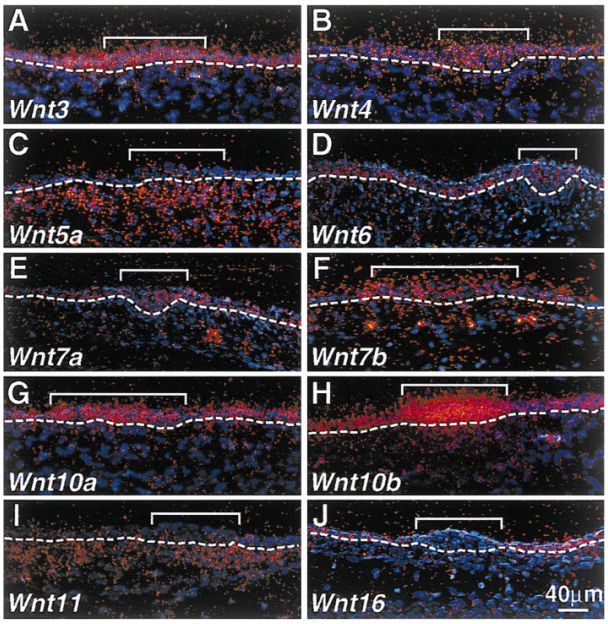

Fig. 3. Expression of *Wnt* genes in embryonic skin. Paraffin sections of trunk skin from embryos at E14.5 were subjected to in situ hybridization with probes for *Wnts 3* (A), *4* (B), *5a* (C), *6* (D), *7a* (E), *7b* (F), *10a* (G), *10b* (H), *11* (I) and *16* (J). The dermal–epithelial junction is indicated by a dashed white line in each panel. Hybridization appears as red grains and nuclei are counterstained with Hoechst dye and appear blue. The positions of hair follicle placodes, indicated by white brackets, were determined by examination of the Hoechst-stained sections without red illumination. *Wnts 3*, *4*, *6*, *7a*, *7b*, *10a*, *10b* and *16* are expressed in the epidermis (panels (A,B,D–H,J)). *Wnt10b* is upregulated strongly and *Wnt10a* is upregulated weakly in hair follicle placodes compared with adjacent interfollicular epidermis (H,G). *Wnt16* is downregulated in placodes compared with interfollicular epidermis (J). *Wnts 5a* and *11* are expressed in the dermis (C,I). Sense control probes for these *Wnt* genes gave only background hybridiza-

reported expression of *Wnt7a* in chick feather follicles (Noramly et al., 1999; Widelitz et al., 1999), indicating a species difference in utilization of this *Wnt*. In contrast, expression of *Wnt10b* is markedly upregulated in hair follicle placodes (Fig. 3H) and specific expression of *Wnt10b* in hair follicles is observed throughout morphogenesis (see below). *Wnt10a* is weakly upregulated in placode epithelium (Fig. 3G) and is also specifically expressed in developing hair follicles at later stages. Specific expression of *Wnt5a* is not observed in hair follicles at the placode stage (Fig. 3C), but expression of this gene is upregulated in follicular cells at later morphogenetic stages (see below). The expression of *Wnts 1*, *2*, *3a*, *5b*, *8a*, *8b* and *15* was not detected in embryonic skin by in situ hybridization.

2.2. Expression of Wnts 10b, 10a and 5a is specifically upregulated in hair follicles at early morphogenetic stages

The induction of hair follicle morphogenesis in mice occurs in successive waves, starting at E14.5 when the formation of the largest, guard hair follicles is initiated. A second wave of follicular morphogenesis that generates smaller, awl hair follicles, is initiated at around E16 and initiation of underfur follicles begins at E18 and continues until birth (Mann, 1962). Since it has been suggested that the formation of different types of hair might be regulated by different signaling pathways (Barsh, 1999), we examined expression of *Wnt* genes in developing follicles at multiple stages between E14.5 and birth (see above). The expression of three *Wnt* genes, *Wnts 10b*, *10a* and *5a*, was found to localize specifically to hair follicles at early morphogenetic stages, with guard hair, awl and underfur follicles showing similar patterns of expression.

The expression of *Wnt10b* is dramatically upregulated in follicular epithelium compared with interfollicular epidermis at the earliest (placode) stage of hair follicle development (Figs. 1A, 3H, 4A,D), consistent with published data (St-Jacques et al., 1998). The placode size and domain of elevated *Wnt10b* expression is significantly larger for guard hair placodes forming at E14.5 than for underfur placodes forming later in development (compare Fig. 4 panels A and D), reflecting the eventual difference in size of these follicles. As morphogenesis proceeds, expression of *Wnt10b* becomes restricted to follicular epithelial cells immediately overlying the dermal condensate (Fig. 4D,G) and subsequently to matrix cells and a cone of cells overlying the dermal papilla that contains precursors of the inner root sheath (Paus et al., 1999) (Fig. 4J).

The expression of *Wnt10a* is slightly upregulated in the placode compared with interfollicular epidermis (Fig. 3G, 4B) and subsequently becomes localized to follicular cells immediately adjacent to the dermal condensate, as well as appearing in the dermal condensate itself (Fig. 4E,H). Once the dermal papilla forms, *Wnt10a* expression fades in the

The expression of *Wnt5a* is seen generally in the dermis at E14.5, when hair follicle placodes first appear (Figs. 3C, 4C), but is specifically upregulated in the dermal condensate at early germ stages (Fig. 4F) and subsequently intensifies further in the dermal condensate and dermal papilla (Fig. 4I,L). At the bulbous peg stage, *Wnt5a* expression also appears at low levels in follicular epithelial cells (Fig. 4L). The expression patterns of *Wnts 10b*, *10a* and *5a* in hair follicle morphogenesis are summarized in Fig. 4M.

2.3. Several Wnt genes are expressed in specific subsets of cells in mature anagen hair follicles

We determined the patterns of expression of *Wnt* genes in mature anagen follicles by in situ hybridization of dorsal skin sections from mice at postnatal day 7 using probes for *Wnts 1*, *2*, *3*, *3a*, *4*, *5a*, *5b*, *6*, *7a*, *7b*, *8a*, *8b*, *10a*, *10b*, *11*, *15* and *16*. Of these, specific expression in hair follicles was not detected for *Wnts 1*, *2*, *5b*, *6*, *7a*, *7b*, *8a*, *8b*, *15* or *16*. The expression of *Wnts 10b* and *10a* was observed in inner root sheath precursors within the matrix, with *Wnt10a* expression also extending to more differentiated inner root sheath cells (Fig. 5A,B). The expression of two additional Class I *Wnt* genes, *Wnts 3a* and *3* (Shimizu et al., 1997; Wolda et al., 1993), appears in postnatal anagen follicles. *Wnt3a* is expressed in differentiating inner root sheath cells, in a pattern partially overlapping with that of *Wnt10a* (Fig. 5C). Mice bearing a partial loss of function mutation in *Wnt3a* are viable and do not have an obvious hair phenotype (Greco et al., 1996), suggesting that the functions of *Wnt3a* in the inner root sheath may be redundant with those of *Wnt10a*. *Wnt3* is expressed in precursors of the hair shaft as they begin to differentiate, consistent with our previous results (Millar et al., 1999) (Fig. 5D). *Wnt4*, which is usually classified as a Class II WNT (Du et al., 1995; Shimizu et al., 1997; Ungar et al., 1995), shows a diffuse pattern of expression in the matrix and in immature hair shaft precursor cells (Fig. 5E). These data suggest *Wnt3* as the strongest candidate for the Class I WNT that activates TOPGAL expression in hair shaft precursor cells. However, since the expression domains of *Wnts 10b*, *10a*, *3a* and *4* lie adjacent to, or include, precursor cells of the hair shaft cortex and cuticle, the WNTs encoded by these genes may also signal to hair shaft precursors.

The expression of *Wnt5a* fades in the dermal papilla of postnatal follicles during anagen and intensifies in subsets of cells of the outer root sheath and outer layers of the inner root sheath (Fig. 5F). *Wnt11*, which like *Wnt5a* encodes a Class II WNT capable of regulating cell movements (Heisenberg et al., 2000; Tada and Smith, 2000; Torres et al., 1996), is expressed in subsets of cells of the outer root sheath and dermal sheath (Fig. 5G). The cells expressing *Wnt5a* and *Wnt11* lie in the same region of the follicle, just above the bulb (compare Fig. 5 panels F and G). Interest-

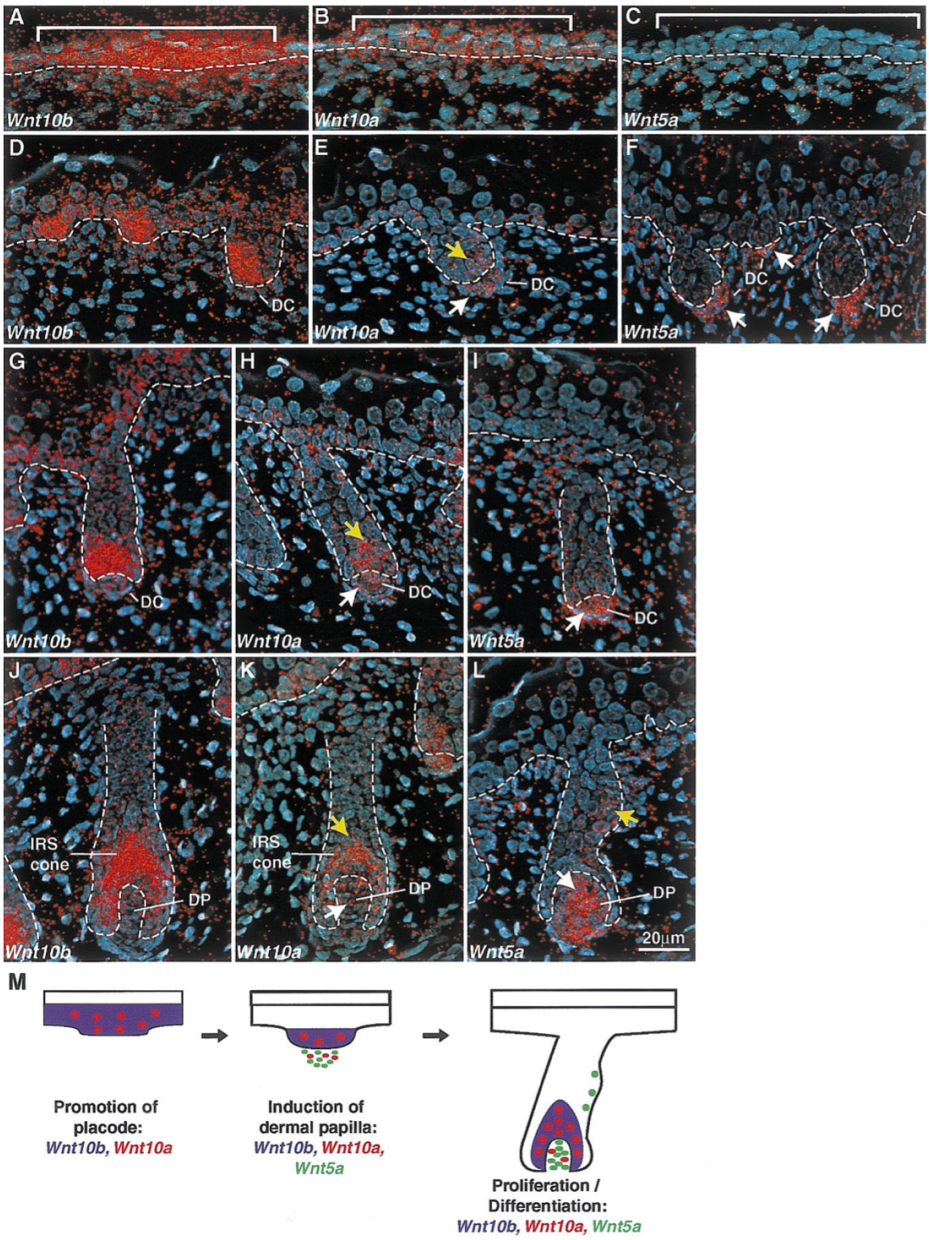

Fig. 4. Expression of *Wnt* genes during hair follicle morphogenesis. In situ hybridization with probes for *Wnts 10b* (A,D,G,J), *10a* (B,E,H,K) and *5a* (C,F,I,L) at E14.5 (A–C) and E18.5 (D–L). The dermal–epithelial junction is indicated by a dashed white line in each panel. Hair follicle placodes in (A–C) are indicated by white brackets. Hybridization appears as red grains and nuclei are counterstained with Hoechst dye and appear blue. The expression of *Wnt10b* is specifically upregulated in the placode compared with adjacent interfollicular epidermis (A). At hair germ (D) and peg (G) stages, expression of *Wnt10b* is seen in follicular epithelial cells immediately adjacent to the dermal condensate. At the bulbous peg stage (J), *Wnt10b* expression localizes to a cone of epithelial cells that are precursors of the inner root sheath (Paus et al., 1999). *Wnt10a* is slightly upregulated in the placode compared with adjacent epidermis (B). At germ (E) and peg (H) stages, *Wnt10a* is expressed in follicular epithelial cells immediately adjacent to the dermal condensate (yellow arrows) and in the dermal condensate itself (white arrows). At the bulbous peg stage (K), *Wnt10a* expression decreases in the dermal papilla (white arrows) and concentrates in inner root sheath precursors (yellow arrows). *Wnt5a* is expressed generally in the dermis at the placode stage (C). At the hair germ (F) and peg (I) stages, *Wnt5a* expression is specifically elevated in the dermal condensate, with more advanced follicles giving the strongest signals (white arrows). At the bulbous peg stage (L), *Wnt5a* is expressed strongly in the dermal papilla (white arrows) and at lower levels in follicular epithelial cells (yellow arrows). Low levels of *Wnt5a* expression remain present in the dermis of E18.5 skin (L). (M) Summary diagram of *Wnt* gene expression in developing hair follicles. Expression patterns of *Wnt10b*, *Wnt10a* and *Wnt5a* are

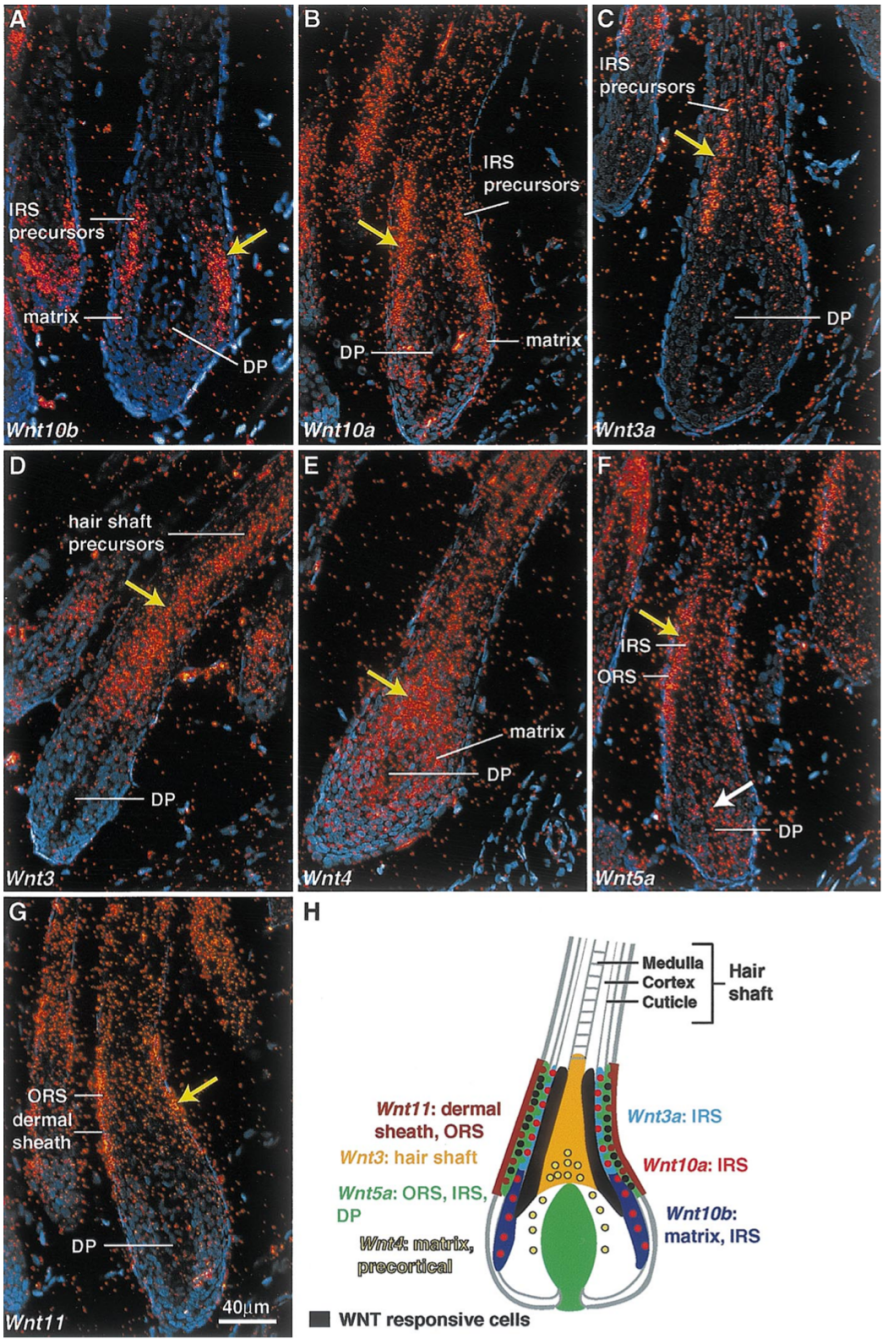

Fig. 5. Expression of *Wnt* genes during anagen. Sections of dorsal skin at postnatal day 7 were hybridized with probes for *Wnts 10b* (A), *10a* (B), *3a* (C), *3* (D), *4* (E), *5a* (F) and *11* (G). (A) *Wnt10b* is expressed in matrix cells that are precursors of the inner root sheath. (B) *Wnt10a* is expressed in matrix cells that are precursors of the inner root sheath and in more differentiated inner root sheath cells. (C) *Wnt3a* is expressed in differentiating inner root sheath cells. (D) *Wnt3* is expressed in hair shaft precursor cells as they differentiate. (E) *Wnt4* is expressed diffusely in the matrix and in the precursors of hair shaft cells lying above the dermal papilla. In the section shown, the hair bulb has not been cut exactly through its center and the middle region of the bulb contains epithelial as well as dermal papilla cells. Examination of additional follicles in which the plane of section bisects the dermal papilla revealed that hybridization of the *Wnt4* probe is to epithelial and not dermal papilla cells (data not shown). (F) *Wnt5a* is expressed at low levels in the dermal papilla (white arrow) and at higher levels in subsets of cells in the outer root sheath and outer layers of the inner root sheath, above the follicle bulb (yellow arrow). (G) *Wnt11* is expressed in subsets of dermal sheath and outer root sheath cells above the follicle bulb. (H) Summary diagram of *Wnt* expression in anagen hair follicles. WNT responsive cells are shown in black and include precursor cells of the hair shaft cortex and cuticle (DasGupta and Fuchs, 1999; Millar et al., 1999) (solid black) and a subset of outer root sheath cells lying above the bulb (Miller et al., 1999) (solid black circles). Hybridization signals are indicated by arrows in panels (A–G). The photographs

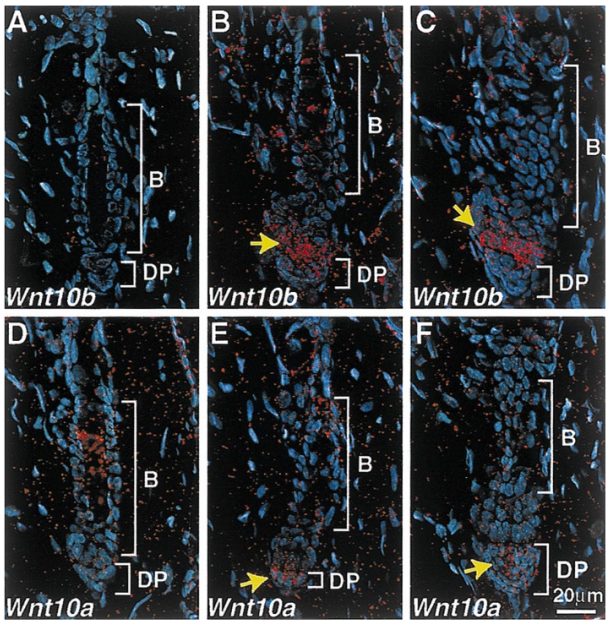

Fig. 6. Expression of *Wnt* genes at anagen onset. Sections of dorsal skin at postnatal day 23 were probed with *Wnts 10b* (A–C) and *10a* (D–F). Telogen stage follicles are shown in panels (A) and (D) and follicles entering anagen are shown in panels (B), (C), (E) and (F). *Wnts 10b* and *10a* are not specifically expressed in the hair follicle during telogen (A,D). At anagen onset, *Wnt10b* is expressed in follicular epithelial cells immediately overlying the dermal papilla (B,C) and *Wnt10a* is weakly upregulated in the dermal papilla (E,F). Hybridization signals are indicated by yellow arrows. The scale bar shown in (F) represents 20 μm. DP, dermal papilla; B, bulge.

signaling pathways (Millar et al., 1999). The effects of *Wnt11* on the control of cell movements appear to be mediated by DVL2 in *Xenopus* (Tada and Smith, 2000). These expression data and the known properties of WNTs 5a and 11 suggest these WNTs as candidates for the signals that control the movements of outer root sheath cells during anagen. The expression patterns of *Wnt* genes in mature anagen follicles are summarized in Fig. 5H.

2.4. Wnts 10b and 10a are expressed at anagen onset

To determine the patterns of *Wnt* gene expression at the onset of a new cycle of hair growth, we carried out in situ hybridization experiments with the probes listed above, using cephalo-caudal strips of dorsal skin from mice at postnatal day 23. At this stage, follicles in posterior regions of the skin are in telogen and more anterior follicles are just entering the first postnatal anagen phase. The expression of *Wnts 10b* and *10a* was detected in follicles at anagen onset (Fig. 6B,C,E,F), but not in resting follicles (Fig. 6A,D), with expression of *Wnt10b* in epithelial cells immediately overlying the dermal papilla and adjacent to the hair follicle bulge (Fig. 6B,C), and expression of *Wnt10a* appearing at low levels in the dermal papilla itself (Fig. 6E,F). None of the other *Wnt* genes examined were specifically expressed in lization at the initiation of follicular morphogenesis, suggesting that morphogenetic molecular mechanisms are re-utilized during the hair growth cycle.

2.5. Wnt5a is not expressed in the dermal condensate of hair follicles lacking SHH

Shh is expressed in the epithelial placode of developing hair follicles and expression of two genes whose expression is positively regulated by SHH, *Patched 1* (*Ptc1*), encoding an SHH receptor, and *Gli1*, encoding a transcriptional mediator of SHH signaling, is observed both in the follicular epithelium and in the dermal condensate (Chiang et al., 1999; St-Jacques et al., 1998). These observations suggest that SHH signals are received both by the epithelium and the mesenchyme of developing follicles. The expression of a stabilized form of β-catenin in the epidermis induces the formation of ectopic hair follicles and *Shh* expression (Gat et al., 1998), and in the absence of epidermal β-catenin *Shh* is not expressed (Huelsken et al., 2001), indicating that *Shh* lies downstream of the canonical WNT signaling pathway in hair follicle morphogenesis. Our finding that transcripts for *Wnt10b* and *Wnt10a* localize to hair follicles at the earliest stage of their formation suggests these *Wnts* as candidate regulators of *Shh* expression. Consistent with this, expression of *Wnt10b* in developing hair follicles is unaffected by lack of SHH (St-Jacques et al., 1998 and Fig. 7A,B).

By analogy with other developmental systems, however,

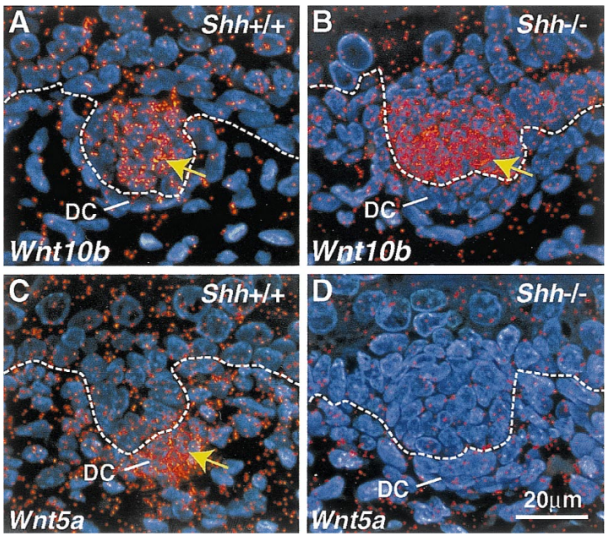

Fig. 7. *Wnt5a* is not expressed in the hair follicles of *Shh*−/− embryos. In situ hybridization of sagittal sections of *Shh*+/+ (A,C) and *Shh*−/− (B,D) littermate embryos at E18.5 with probes for *Wnt10b* (A,B) and *Wnt5a* (C,D). Expression of *Wnt10b* is seen in the epithelial cells of germ stage hair follicles in *Shh*+/+ and *Shh*−/− skin (A,B) (yellow arrows). Expression of *Wnt5a* is seen in the dermal condensate of germ stage hair follicles in *Shh*+/+ skin (C) (yellow arrow) but is absent from the dermal condensate in *Shh*−/− skin (D). The dermal–epithelial junction is marked by a dashed white line in each panel. Photographs in all panels were taken at the

WNT genes may also be targets of SHH in hair follicles (Hammerschmidt et al., 1997; Liu et al., 2000). Since *Wnt5a* is expressed slightly later in hair follicle morphogenesis than *Wnt10b* and *Shh*, we wondered whether it is regulated by SHH. To test this, we examined the expression of *Wnt5a* in *Shh*−/− embryos and control littermate *Shh*+/+ embryos at E14.5, E16.5 and E18.5. In both *Shh*+/+ and *Shh*−/− embryos, placodes were visible at E14.5 and germ stage follicles at E16.5 and E18.5. Consistent with published results, later stages of follicle development (peg and bulbous peg) were observed only in *Shh*+/+ embryos, indicating that follicle morphogenesis is abnormal after the germ stage in the absence of SHH (Chiang et al., 1999; St-Jacques et al., 1998). The expression of *Wnt10b* was used as a positive control for hybridization and was observed in *Shh*+/+ and *Shh*−/− follicles at all stages examined (Fig. 7A,B). Specific expression of *Wnt5a* was not observed in placode stage hair follicles in wild-type or *Shh*−/− mutant embryos at E14.5, consistent with our previous results (above and data not shown). At E16.5 and E18.5, expression of *Wnt5a* was observed in the hair follicle dermal condensates of follicles in *Shh*+/+ but not *Shh*−/− skin (Fig. 7C,D). The dermal condensate was clearly present in germ stage *Shh*−/− hair follicles (Fig. 7B,D); thus absence of *Wnt5a* expression is not due to absence of the cells that normally express *Wnt5a*. These results indicate that *Wnt5a* is a target of SHH in hair follicle morphogenesis and suggest a model in which *Wnt* genes lie both upstream and downstream of *Shh* in this developmental process (Fig. 8).

3. Discussion

3.1. WNTs 10b, 10a and 5a are candidates for key morphogenetic signals

Although multiple *Wnt* genes are expressed in the surface epithelium of the embryo, transcripts for only two of these, *Wnts 10a* and *10b*, become specifically localized to hair follicle placodes. Since WNT10b causes mammary tumors in mice, stabilization of β-catenin in preadipocytes and partial axis duplication in *Xenopus* embryos, it may be classified as a Class I WNT (Ishikawa et al., 2001; Lane and Leder, 1997; Ross et al., 2000). The signaling properties of WNT10a have not yet been determined, but at the sequence level *Wnts 10a* and *10b* encode closely related proteins (Wang and Shackleford, 1996), making it likely that WNT10a is also a Class I WNT. WNTs 10a and 10b are therefore strong candidates for the signals that cause accumulation of nuclear β-catenin and activation of TOPGAL in the epithelium and mesenchyme at early stages of follicular development (DasGupta and Fuchs, 1999; Noramly et al., 1999). In the absence of epidermal β-catenin, placodes fail to form, whereas expression of a stabilized form of β-catenin in the epithelium causes the formation of ectopic hair follicles, indicating that the canonical WNT signaling pathway acts to promote formation of the placode and induction of the dermal condensate (Gat et al., 1998; Huelsken et al., 2001). WNTs10a and 10b may therefore comprise part of the 'first epithelial signal' operating in hair follicle morphogenesis.

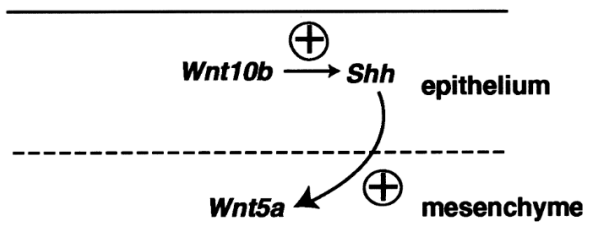

Fig. 8. Model for the interactions of *Wnt10b*, *Shh* and *Wnt5a* in early hair follicle morphogenesis. According to this model, *Wnt10b* promotes placode fate by mechanisms that include positive regulation of *Shh* gene expression. SHH expressed in the placode induces expression of *Wnt5a* in the dermal

Wnt5a can function as a Class II *Wnt* (Du et al., 1995; Moon et al., 1993; Shimizu et al., 1997; Torres et al., 1996) although it is also capable of directing the canonical pathway in the presence of an appropriate Frizzled receptor (He et al., 1997; Umbhauer et al., 2000). *Wnt5a* may therefore have functions in hair follicles other than or in addition to those suggested by TOPGAL expression and the effects of loss and gain of function mutations in Class I pathway genes. Its expression pattern in developing hair follicles suggests that it is not involved in the initial positioning of follicles, but may form a component of the 'second dermal signal', directing the proliferation of overlying epithelial cells. Such a role would be consistent with the established properties of *Wnt5a*, which has been shown to be required for the proliferation of limb bud and snout progenitor cells (Yamaguchi et al., 1999).

In contrast to its expression in developing chick feather follicles (Noramly et al., 1999; Widelitz et al., 1999), *Wnt7a* is not specifically expressed in developing hair follicles at any stage of morphogenesis. This observation may reflect a fundamental difference in the biology of feather and hair follicles. Alternatively, the functions of *Wnt7a* in feather buds may be performed by a different member of the *Wnt* family, such as *Wnt10a* or *Wnt10b*, in developing hair follicles.

3.2. Wnt gene expression patterns suggest multiple roles for WNT signaling in mature hair follicles

Several lines of evidence indicate that, in addition to roles in hair follicle morphogenesis and at anagen onset, signaling by Class I WNTs may regulate differentiation of the hair shaft. Firstly, the few hairs that develop in *Lef1*-deficient mice appear to be incompletely keratinized (Kratochwil et al., 1996). Secondly, the WNT-responsive TOPGAL repor-

Millar et al., 1999). Thirdly, ectopic expression of *Wnt3* in the follicular outer root sheath causes fragility of the hair shaft (Millar et al., 1999). The results presented here indicate that *Wnt3* is expressed in the most differentiated hair shaft precursor cells during anagen, making it the strongest candidate for a regulator of hair shaft differentiation. However, the expression pattern of *Wnt3* overlaps with that of *Wnt4* in less differentiated hair shaft precursors and *Wnts 10a*, *10b* and *3a* are expressed in inner root sheath cells adjacent to hair shaft precursors, suggesting possible functional redundancy between these *Wnts* and *Wnt3* in mature follicles.

It has recently been demonstrated that during the anagen phase in mature follicles, epithelial progenitor cells in the outer root sheath migrate from the bulge region to the hair bulb where they proliferate and subsequently begin to differentiate into inner root sheath and hair shaft precursors (Oshima et al., 2001; Taylor et al., 2000). These cell movements are likely to be important for a normal rate of hair growth. Signals controlling cell movements have been studied in *Xenopus* and zebrafish embryos. These experiments revealed that cell migration during gastrulation is regulated by WNTs 5a and 11 via a non-canonical WNT signaling pathway (Djiane et al., 2000; Heisenberg et al., 2000; Moon et al., 1993; Sokol, 2000; Tada and Smith, 2000). Signaling by WNT11 requires the WNT effector DVL, but not β-catenin and is similar to a pathway regulating planar polarity in *Drosophila* embryos (Adler, 1992; Djiane et al., 2000; Heisenberg et al., 2000; Sokol, 2000; Tada and Smith, 2000; Wallingford et al., 2000). A characteristic of these pathways is that disruption of cell polarity and movement occurs as a result of either inhibiting or stimulating signaling (Djiane et al., 2000; Krasnow and Adler, 1994; Wallingford et al., 2000). We find that *Wnt 5a* and *Wnt11* are expressed in outer layers of anagen hair follicles in cells that lie in the neck of the follicle between the bulge and the hair bulb. DVL2 is expressed in the outer root sheath in this same region of the follicle and overexpression of DVL2 in the outer root sheath causes a phenotype of short hair that is not the result of decreased follicle cell proliferation or altered control of the hair growth cycle (Millar et al., 1999). These results suggest that cell movements during anagen may be regulated by WNTs 5a and 11, via DVL2. Experiments in which the functions of these WNTs are increased or inhibited in the outer root sheath, combined with recently described methods for tracking cell movements in hair follicles (Taylor et al., 2000), may be used to test this hypothesis.

3.3. WNT expression patterns are similar at the onset of morphogenesis and postnatal anagen

Although several factors capable of inducing a new cycle of hair growth in resting follicles have been identified (Paus molecular level. It has been proposed that the initiating signal for hair growth arises from the dermal papilla and instructs epithelial stem cells in the bulge region of the follicle to proliferate transiently (Cotsarelis et al., 1990). This hypothesis is supported by the observation that the first postnatal hair growth cycle is not initiated in the *hairless* (*hr*) mutant mouse, in which the dermal papilla loses contact with the epithelial portion of the follicle during catagen (Cotsarelis et al., 1990; Panteleyev et al., 1999). The transient expression of the TOPGAL reporter gene in bulge cells at anagen onset suggests that WNTs may be involved in triggering a new cycle of hair growth (DasGupta and Fuchs, 1999). In support of this hypothesis, onset of the first postnatal anagen does not occur in mice in which the β-catenin gene is progressively deleted in the epidermis and follicular epithelium (Huelsken et al., 2001). We find that *Wnt10a* is expressed in the dermal papilla at anagen onset, while *Wnt10b* is expressed in adjacent epithelial cells in the lower part of the follicle. These results suggest WNT10a as a possible component of an initiating signal from the dermal papilla and identify WNTs 10a and 10b as the strongest candidates for the WNTs that induce TOPGAL expression in the bulge. The timing and location of expression of *Wnt10b* correlate with the migration of progenitor cells to the lower part of the hair follicle (Oshima et al., 2001; Taylor et al., 2000) suggesting that WNT10b is expressed in these 'activated' progenitor cells. These results also identify WNT10b as the strongest candidate for the epithelial signal that maintains the inductive properties of follicular dermal cells (Kishimoto et al., 2000).

3.4. Wnt5a is a target of SHH in hair follicle morphogenesis

SHH is not required for the positioning of follicles, but plays essential roles in the regulation of follicular proliferation and formation of the dermal papilla (Chiang et al., 1999; St-Jacques et al., 1998). Previous data have indicated that expression of *Shh* in hair follicles is regulated by canonical WNT signaling (Gat et al., 1998; Huelsken et al., 2001) and our results suggest WNTs 10a and 10b as the most likely candidates for WNTs that control *Shh* expression in hair follicle morphogenesis (see model in Fig. 8). However, *Wnt* genes are also targets of SHH in several developmental systems (Hammerschmidt et al., 1997; Liu et al., 2000) and consistent with this observation, we find that expression of *Wnt5a* in developing hair follicles requires SHH. This result suggests that WNT5a may mediate some of the effects of SHH in hair follicle morphogenesis, a hypothesis supported by the fact that both WNT5a and SHH are capable of regulating proliferation (Chiang et al., 1999; St-Jacques et al., 1998; Yamaguchi et al., 1999). Since later stages of hair follicle morphogenesis are abnormal in *Shh*−/− mutants (Chiang et al., 1999; St-Jacques et al., 1998), we were unable to address the question of

expressed in inner root sheath cells in anagen follicles (Millar, 1997).

Our finding that *Wnt5a* is a target of SHH signaling in hair follicles has important implications for the study of BCC, a human skin tumor that occurs with high frequency in Caucasian populations. BCC results from inappropriate activation of the SHH pathway in epidermal cells and is frequently associated with mutations in the gene encoding the SHH receptor PTC1 (Hahn et al., 1996; Johnson et al., 1996; Unden et al., 1996). Like developing hair follicles, BCCs show elevated expression of *PTC1* and *GLI1*, which encodes a transcriptional effector of SHH signaling (Dahmane et al., 1997; Nagano et al., 1999). In addition to activation of the SHH signaling pathway, BCCs share many common characteristics with immature hair follicles, including similar histology, ultrastructure and patterns of keratin gene expression (Kumakiri and Hashimoto, 1978; Markey et al., 1992), suggesting that SHH activates the same downstream target genes in BCCs and hair follicles. BCC can be mimicked in transgenic mice by over-expression of *Shh*, *Gli1* or *Gli2* in the epidermis (Grachtchouk et al., 2000; Nilsson et al., 2000; Oro et al., 1997) and *Wnt* gene expression is directly regulated by SHH via GLI transcription factors in *Drosophila* and zebrafish embryos (Liu et al., 2000; Von Ohlen et al., 1997). However, *Wnt* targets of the SHH pathway in BCC have not been identified. Given the similarity of BCC to immature hair follicles, our results predict that *Wnt5a* is upregulated in BCC. Nuclear localization of β-catenin is not observed in BCC (Boonchai et al., 2000) consistent with classification of WNT5a as a Class II WNT (Du et al., 1995; Moon et al., 1993; Shimizu et al., 1997; Torres et al., 1996).

4. Experimental procedures

4.1. RT-PCR

For RT-PCR experiments, we dissected dorsal skin from FVB/N mouse embryos at embryonic day 14.5 (E14.5), extracted RNA using Trizol (Gibco BRL, Rockville, MD, USA) and synthesized cDNA using 5 μg of RNA in a volume of 40 μl. Two microliters of each reverse transcription reaction was subjected to 30 rounds of PCR using specific primers for mouse *Wnt* genes. Each experiment was repeated on skin from at least three embryos. Primers were designed to amplify non-conserved regions of the cDNAs. Eighteen base pair (bp) primers were used to amplify the following sequences whose Genbank accession numbers are shown in brackets: *Wnt1*, 1311–1711 (M11943); *Wnt2b*, 1034–1488 (AF070988); *Wnt3a*, 1161–1581 (X56842); *Wnt3*, 1103–1514 (M32502); *Wnt4*, 1–430 (M89797); *Wnt5a*, 522–870 (M89798); *Wnt5b*, 4–294 (M89799); *Wnt6*, 1–298 (M89800); *Wnt7a*, 501–1050 1589–1943 (U6169); *Wnt10b*, 176–343 (NM011718.1); *Wnt11*, 849–1340 (X70800); *Wnt15*, 1–299 (AF031169). Primers for amplification of exons 3 and 4 of *Wnt2* cDNA were identical to those described in Monkley et al. (1996).

4.2. In situ hybridization

We performed in situ hybridization with ^{35}S-labeled probes on sectioned, paraffin-embedded tissue, as previously described (Millar et al., 1999). Balb/c postnatal mice and FVB/N embryos from timed natural matings were used as the sources of wild-type tissue. *Shh*−/− embryos and control *Shh*+/+ littermate embryos were obtained from natural matings of *Shh*+/− knockout mice (Chiang et al., 1996) maintained on a CD1 background. *Shh* mutant and control embryos were genotyped by PCR as described in Chiang et al. (1996). We dissected anterior–posterior strips of skin from the mid-dorsum of postnatal animals prior to fixation. A single slit was made in the mid-ventrum of all embryos and embryos aged E15.5 and older were also decapitated, to allow for easier penetration of the fixative. Fixation was carried out overnight in 4% paraformaldehyde at 4°C, prior to dehydration and paraffin embedding. Embryos were sectioned sagitally and dorsal skin strips were sectioned parallel to the anterior–posterior axis. Probes for *Wnts 4*, *5a*, *6*, *7a* and *7b* were identical to those described in Parr et al. (1993); *Wnt11* probe was identical to that described in Kispert et al. (1996); *Wnt5b* probe was identical to that described in Gavin et al. (1990); and probes for *Wnt3* and *Wnt3a* were identical to those described in Roelink and Nusse (1991). Probes for *Wnts 10a* and *10b* were synthesized from cDNA clones described in Wang and Shackleford (1996). Probes for *Wnts 1*, *2*, *8a*, *8b* and *15* were synthesized by RT-PCR of E14.5 mouse embryo RNA using the primers described above, and a probe for *Wnt16* was synthesized by the same method, using 18 bp primers to amplify nucleotides 439–869 of *Wnt16* cDNA (Genbank accession number AF 172064); sequences for binding of T7 RNA polymerase were added to the 3′ primers to create templates for synthesis of antisense probes and to the 5′ primers for creating sense probe templates. Sections were counterstained with 2 μg/ml Hoechst (Sigma, St. Louis, MO, USA), mounted in 50% w/v Canada balsam in methyl salicylate (Sigma) and photographed using an Olympus Bx60 microscope with an MVI Darklite stage adaptor and Photometrics Coolsnap digital camera.

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