Expression of *Frizzled* Genes in Developing and Postnatal Hair Follicles

Seshamma T. Reddy,*[1] Thomas Andl,*[1] Min-Min Lu,† Edward E. Morrisey,† and Sarah E. Millar*
*Departments of Dermatology and Cell and Developmental Biology and †Cardiology Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Embryonic hair follicle development and postnatal hair growth rely on intercellular communication within the epithelium and between epithelial and mesenchymal cells. Several members of the WNT family of paracrine intercellular signaling molecules are expressed in specific subsets of cells in developing and mature mouse hair follicles, suggesting them as candidates for some of the intercellular signals that operate in these organs. As WNT ligands activate several different signaling pathways, they may play multiple and complex roles in developing and postnatal skin. To begin to investigate these functions, we have used *in situ* hybridization to identify cells that express *Frizzled* (*Fz*) WNT receptor genes, and so are potentially receptive to WNT ligands. We find that several *Fz* genes are specifically expressed at sites of known activity of the WNT/β-catenin signaling pathway, allowing us to identify candidate receptors for canonical WNT ligands important in appendage development. The expression of additional *Fz* genes is specifically elevated at locations and developmental stages other than those that display WNT/β-catenin pathway activity, suggesting that signaling through alternate WNT pathways may contribute to the development and function of skin and hair.

Key words: *Frizzled*/hair follicle/mouse/*Wnt*
J Invest Dermatol 123:275–282, 2004

Hair follicles develop via extensive interactions between cells of the surface ectoderm and underlying dermal cells (Hardy, 1992; Millar, 2002). The first morphological sign of hair follicle formation is the development of a regular array of placodes, or local thickenings, in the surface ectoderm, which are formed in response to signals from the dermis (Hardy, 1992). Signaling from each placode to the dermis causes the formation of a cluster of mesenchymal cells, known as a dermal condensate (DC) (Hardy, 1992). Reciprocal signaling from the DC to the epithelium results in the proliferation and downgrowth of epithelial cells into the dermis, forming a hair germ. The hair germ continues to elongate, to form a hair peg, and at this stage epithelial cells begin to surround the DC, which develops into the dermal papilla (DP) of the hair follicle (the "bulbous peg" stage). The follicular epithelium subsequently differentiates to form several concentric cell layers, which include the medulla, cortex, and cuticle of the hair shaft, and the cortex, Huxley's layer, and Henle's layer of the inner root sheath (IRS) that molds the developing hair shaft as it emerges from the follicle (Sperling, 1991). The IRS is surrounded by an outer root sheath (ORS) that is contiguous with the epidermis, and the follicle is bound by a connective tissue sheath. Production of the hair shaft and IRS is dependent on signaling between the DP and the follicular epithelium (Oliver and Jahoda, 1988), and is also likely to require communication between cells in different epithelial layers of the follicle (Millar *et al*, 1999; Lin *et al*, 2000).

After birth, hair follicles undergo successive cycles of growth (anagen), regression (catagen), and rest (telogen) (Dry, 1926; Muller-Rover *et al*, 2001). At the onset of a new growth cycle, stem cells in the permanent, bulge, region of the follicle are stimulated to divide, possibly by signals from the DP (Oliver and Jahoda, 1988; Cotsarelis *et al*, 1990). Stem cell progeny move to the lower part of the follicle and proliferate rapidly, forming the hair follicle matrix (Taylor *et al*, 2000; Oshima *et al*, 2001). Progeny of the matrix cells cease dividing and differentiate along specific pathways to form a new hair shaft and IRS, and the old hair is eventually shed. The movement of progenitor cells from the bulge to the matrix has been shown to continue throughout anagen in vibrissa follicles (Oshima *et al*, 2001); however, the mechanisms controlling these movements are unknown.

Among the intercellular signals active in developing and postnatal follicles, secreted WNT proteins appear to play particularly crucial roles (Millar, 2002). WNTs form a large family with 19 members in humans and mice (see http://www.stanford.edu/∼rnusse/wntwindow.html). These bind to their receptors, the Frizzled (FZ) proteins, that also form a large family with ten members in humans and mice, to activate several different pathways that control cellular proliferation, fate, shape, and movements (Moon *et al*, 2002; Veeman *et al*, 2003). In the canonical WNT/β-catenin pathway (Moon *et al*, 2002), WNT ligands bind to low-density

Abbreviations: DC, dermal condensate; DP, dermal papilla; DSH, Dishevelled; FZ, Frizzled; IRS, inner root sheath; LDL, low-density lipoprotein; ORS, outer root sheath

[1]These authors contributed equally to this study.

lipoprotein (LDL) receptor-related protein (LRP) 5/6 co-receptors as well as to an FZ receptor, causing activation of the intracellular protein Dishevelled (DSH), and inhibiting the function of a complex of proteins that normally targets cytoplasmic β-catenin for degradation. As a consequence, β-catenin accumulates in the cytoplasm, translocates to the nucleus, and forms transcriptional complexes with members of the lymphocyte enhancer factor (LEF)/T cell factor (TCF) family of DNA binding factors, resulting in the activation of target genes (Moon *et al*, 2002).

An alternate, non-canonical, WNT signaling pathway controls planar polarity in Drosophila embryos, and is required for planar polarity of cochlear hair cells in the mouse inner ear, and for the morphogenetic movements that occur during gastrulation in vertebrate embryos (Veeman *et al*, 2003). In non-canonical WNT signaling, binding of WNT to FZ receptors is enhanced by Knypek, a member of the glypican family of heparan sulfate proteoglycans (Topczewski *et al*, 2001), but does not involve LRP5/6. Non-canonical WNT signaling activates DSH, but utilizes different domains of the DSH protein than those involved in WNT/β-catenin signaling (Axelrod *et al*, 1998). In addition, non-canonical WNT signaling does not require β-catenin or LEF/TCF factors; instead, DSH activates Rho1 via a bridging molecule Daam1 (Habas *et al*, 2001). Activated DSH can cause calcium flux and activation of the calcium-sensitive kinases protein kinase C and CamKII (Slusarski *et al*, 1997; Kuhl *et al*, 2000). Whether Rho1 activation and activation of calcium flux occur in the same pathway, or represent different branches of the non-canonical FZ–DSH pathway, however, is currently unclear (Veeman *et al*, 2003). In some contexts, non-canonical WNT signaling can act to antagonize signaling through the WNT/β-catenin pathway (Weidinger and Moon, 2003).

Certain WNT ligands appear to activate preferentially either the WNT/β-catenin pathway or non-canonical WNT signaling; for instance, WNT5A is generally described as an activator of non-canonical signaling (e.g., Weidinger and Moon, 2003), whereas WNT1 activates canonical signaling (Korinek *et al*, 1998). This distinction is context-dependent rather than absolute; for instance, WNT5A can activate the canonical pathway in Xenopus embryos when FZ5 is expressed (He *et al*, 1997), and WNT1 activates RhoA in 293T cells (Habas *et al*, 2001). Similarly, over-expression of FZ1 in Xenopus embryo animal cap explants activates the canonical pathway (Yang-Snyder *et al*, 1996), whereas over-expression in human 293T cells activates RhoA (Habas *et al*, 2001), and FZ7 has been implicated in both canonical and non-canonical pathways in different systems (Sumanas *et al*, 2000; Habas *et al*, 2001). Thus, activation of canonical *versus* non-canonical signaling is likely to depend on the precise pairing of WNT and FZ ligands, as well as on the presence of particular co-receptors, endogenous secreted inhibitors (Zorn, 2001; Pandur *et al*, 2002; Kawano and Kypta, 2003), and intracellular factors.

Investigations of the possible functions of non-canonical WNT signaling in skin and hair follicles have not been Birchmeier, 2001; Andl *et al*, 2002). Analysis of the *in vivo* expression of a TOPGAL WNT reporter transgene, in which the *lacZ* gene is placed under the control of multiple LEF/TCF binding sites and a minimal promoter, reveals activity of the canonical WNT pathway in both the epithelium and the mesenchyme of developing hair follicles (DasGupta and Fuchs, 1999), consistent with the involvement of WNT signals in the cross-talk between follicular epithelial and mesenchymal cells.

Roles for canonical WNT signaling in postnatal hair follicles are suggested by the finding that the TOPGAL WNT/β-catenin reporter transgene is expressed in hair shaft precursor cells (DasGupta and Fuchs, 1999). A second type of WNT/β-catenin reporter transgene, BATgal, containing the promoter regions of the Xenopus WNT target gene Siamois fused to *lacZ*, is additionally expressed in the hair follicle matrix and DP of some hair follicles, suggesting that these are also sites of WNT/β-catenin signaling activity (Maretto *et al*, 2003). TOPGAL is reportedly expressed in hair follicle stem cells at anagen onset (DasGupta and Fuchs, 1999), suggesting that activation of WNT signaling might be one factor controlling the telogen–anagen transition. This hypothesis is consistent with the observation that progressive loss of epithelial β-catenin causes failure of onset of the first postnatal anagen (Huelsken *et al*, 2001).

Multiple *Wnt* genes are expressed in developing and mature hair follicles, including *Wnt*s known to be capable of activating the WNT/β-catenin pathway and several *Wnt*s that initiate non-canonical signaling in most developmental contexts (Reddy *et al*, 2001). As discussed above, the specificity of function of the different WNT proteins is likely to be determined in part by the availability of the various FZ receptors. However, with the exception of *Fz3*, which is expressed at several stages of hair follicle development (Hung *et al*, 2001), the *Fz* genes expressed in hair follicles are unknown. To address this question, we have carried out RT-PCR and *in situ* hybridization experiments to examine the expression profile of the known *Fz* genes in developing and postnatal mouse skin. We find that several *Fz* genes are expressed at sites of canonical WNT activity in developing and postnatal hair follicles, allowing us to identify candidate receptors for canonical WNT signals. In addition, certain *Fz* genes are expressed at locations and developmental stages that correlate with the expression of non-canonical *Wnt* genes and do not display WNT/β-catenin reporter activity in transgenic mouse assays. These results suggest that WNT signaling through non-canonical pathways may contribute to the regulation of hair follicle development and hair growth.

Results and Discussion

Several *Fz* genes are specifically expressed in embryonic skin at the time of onset of hair follicle induction To identify *Fz* genes expressed in embryonic skin at the time of onset of hair follicle induction, we carried out RT-PCR

remove any contaminating genomic DNA, and control experiments in which the reverse transcriptase enzyme was omitted were performed for each primer pair and RNA preparation to ensure that PCR products did not arise from amplification of genomic DNA. Before experiments were carried out on embryonic skin RNA, each primer pair was tested on RNA isolated from whole embryos to check that a product of the appropriate size could be amplified. The expression of all of these genes was detected in E14.5 skin by this assay (Fig 1).

To confirm expression of *Fz1*, *Fz2*, *Fz4*, *Fz5*, *Fz6*, *Fz7*, *Fz8*, and *Fz9*, and determine the cellular localization of expression within developing skin, probes for these genes, and an additional, more recently cloned *Fz*, *Fz10*, were used for *in situ* hybridization experiments with sectioned embryos at E14.5. *Fz3* expression in developing skin has previously been described in detail (Hung *et al*, 2001), and was not examined further here. Probes were designed to hybridize to regions of *Fz* cDNAs that did not contain regions of high homology to other *Fz* genes, and would not cross-hybridize with secreted Frizzled-related protein (*sFrp*) mRNAs that are similar to 5′, but not 3′, regions of *Fz* mRNAs (Rattner *et al*, 1997). In most cases, probes were complementary to non-conserved 3′ coding regions or 3′ non-coding regions of *Fz* genes. Hybridization to internal embryonic organs provided a positive control for each probe. Sense probes were used as negative controls. All of these *Fz* genes were expressed at low levels throughout the embryo. Markedly elevated expression in embryonic skin at E14.5 was only observed, however, for *Fz1*, *Fz6*, and *Fz10*. Of these genes, *Fz6* was predominantly expressed in the epidermis, but was not expressed within the epithelium of developing placode and germ stage hair follicles (Fig 2*B*). *Fz1* was expressed at low levels in the dermis, and was specifically elevated in hair follicle placodes (Fig 2*A*). Elevated expression of *Fz10* was confined to developing hair follicles, where it was expressed both in the placode and in the DC (Fig 2*C*). In addition, *Fz2* showed very slightly elevated expression in the placodes and DC of developing hair follicles (data not shown).

These data are summarized and compared with the previously described expression of *Wnt* genes in developing hair follicles at the placode stage (Reddy *et al*, 2001) in Fig 4 (*left diagram*). Expression of *Fz1* and *Fz10* in the placode

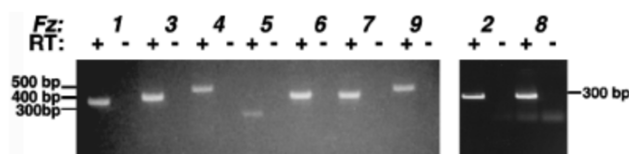

Figure 1
RT-PCR analysis indicates that multiple members of the *Fz* gene family are expressed in embryonic mouse skin. RNA was extracted from the dorsal skin of single mouse embryos at E14.5 and cDNA was synthesized by RT. *Fz* cDNA fragments were amplified using specific primers. PCR products of the expected sizes were amplified using

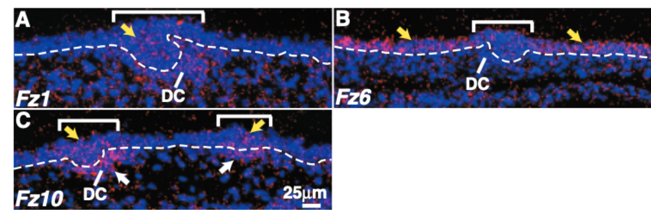

Figure 2
Expression of *Fz* genes in embryonic skin and developing hair follicles at the time of hair follicle initiation. Sagittal paraffin sections of E14.5 mouse embryos were subjected to *in situ* hybridization with probes for *Fz1* (*A*), *Fz6* (*B*), and *Fz10* (*C*). Hybridization appears as red grains and is indicated by arrows. Nuclei are counterstained with Hoechst 33258 dye and appear blue. Developing hair follicles are indicated by brackets. The dermal–epithelial junction is indicated by a dashed white line in each panel. *Fz1* is expressed at low levels in the dermis and is slightly elevated in the follicle epithelium (*A*) (*yellow arrow*). *Fz6* is expressed in the interfollicular epithelium (*yellow arrows*) (*B*) but is downregulated in hair follicle placodes. *Fz10* expression is elevated in the epithelium (*yellow arrows*) and DC (*white arrows*) of developing hair follicles (*C*). Sense control probes for these *Fz* genes gave only background hybridization. The photographs in all panels were taken at the same magnification. *Scale bar* = 25 μm (panel *C*).

and *Fz10* in the developing DC correlates with expression of *Wnt10a* and *Wnt10b* in the placode. WNT10B, FZ1, and FZ10 are all known to be capable of activating canonical WNT signaling (Yang-Snyder *et al*, 1996; Kawakami *et al*, 2000b; Ross *et al*, 2000; Malbon *et al*, 2001; Terasaki *et al*, 2002). The signaling properties of *Wnt10a* have not been described, but its high degree of sequence similarity to, and overlapping expression with, *Wnt10b* suggests that it may have similar properties (Wang and Shackleford, 1996; Reddy *et al*, 2001). These data suggest that canonical WNT signaling is likely activated in the placodes and DC of developing hair follicles by WNTs 10A and 10B, expressed in and secreted from epithelial cells, binding to FZ1 and FZ10, expressed in the placode epithelium and DC.

Interestingly, *Fz6* is expressed in the embryonic interfollicular epidermis, overlapping with the previously described expression of *Fz3* (Hung *et al*, 2001). This expression persists in postnatal epidermis (see below) (Hung *et al*, 2001). *In vivo* assays indicate that the canonical WNT/β-catenin signaling pathway is not active in embryonic or postnatal interfollicular epidermis (DasGupta and Fuchs, 1999; Maretto *et al*, 2003). *Fz3* and *Fz6*, however, are able to stimulate non-canonical WNT signaling (Sheldahl *et al*, 1999), and the interfollicular epidermis displays expression of several *Wnt*s, including *Wnt4* and *Wnt6*, that can activate non-canonical signaling (Wong *et al*, 1994; Reddy *et al*, 2001; Westfall *et al*, 2003). In addition, two non-canonical *Wnt* genes, *Wnt5a* and *Wnt11*, are expressed in the underlying dermis (Reddy *et al*, 2001). These data raise the possibility that non-canonical WNT signaling is active in interfollicular epidermis.

Additional *Fz* genes are expressed as hair follicles undergo morphogenesis To examine the profile of *Fz* gene expression at later stages of embryonic hair follicle

Expression of Frizzled Genes in Developing and Postnatal Hair Follicles | Elsevier Enhanced Reader 9/24/19, 1:20 PM

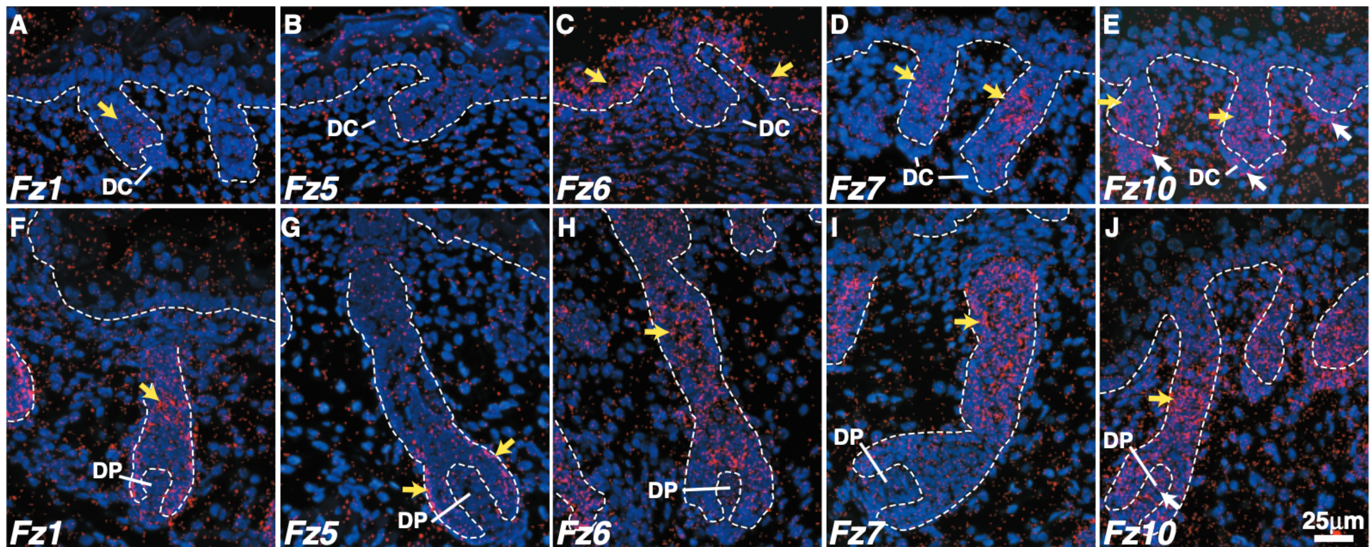

Figure 3
***Fz* genes are expressed throughout hair follicle morphogenesis.** Sagittal paraffin sections of mouse embryos at E16.5 (*C*) or E18.5 (*A, B, D–J*) were subjected to *in situ* hybridization with probes for *Fz1* (*A, F*); *Fz5* (*B, G*); *Fz6* (*C, H*); *Fz7* (*D, I*); and *Fz10* (*E, J*). Secondary hair follicles at the germ and peg stages are shown in (*A, B, D, E*); a primary hair follicle at the early peg stage is shown in (*C*); primary hair follicles at the bulbous peg stage are shown in (*F–J*). Nuclei are counterstained with Hoechst 33258 dye and appear blue. The dermal–epithelial junction is indicated by a dashed white line in each panel. At the late germ–early peg stages, *Fz1* is expressed at very low levels in the follicle epithelium (*yellow arrow*) (*A*); *Fz5* is not specifically expressed in the follicle (*B*); *Fz6* is expressed in the epidermis (*yellow arrows*) and is downregulated in follicle epithelium (*C*); *Fz7* is expressed in proximal follicle epithelium (*arrows*) (*D*); and *Fz10* is specifically expressed in the follicle epithelium (*yellow arrows*) and DC (*white arrows*) (*E*). At the bulbous peg stage, expression of *Fz1* and *Fz5* localizes to the developing ORS (*arrows*) (*F, G*); *Fz6* is expressed in central hair follicle epithelial cells (*arrow*) as well as in the epidermis (*H*); *Fz7* expression intensifies in central, proximal follicular epithelial cells (*arrow*) (*I*); and *Fz10* expression is maintained in the DP (*white arrow*) and follicle epithelium (*yellow arrow*) (*J*). The smaller follicles in (*J*) (*right side*) are secondary follicles at the early peg stage. Sense control probes for these *Fz* genes gave only background hybridization. The photographs in all panels were taken at the same magnification. *Scale bar* = 25 μm (panel *C*). DC, dermal condensate; DP, dermal papilla.

follicles at these stages; however, *Fz4* transcripts localize to the muscle layer underlying the dermis. *Fz2* expression is very slightly elevated within the epithelium and DC of germ and peg stage follicles, and is very weakly expressed in the epithelium above the bulb in bulbous peg stage follicles (data not shown). At the germ and peg stages, *Fz1*, and, more prominently, *Fz10*, are expressed in epithelial cells, and *Fz10* is also expressed in DC (Fig 3*A, E*). Expression of *Fz1* in follicular epithelium, and *Fz10* in follicular epithelial and dermal cells, is maintained at the bulbous peg stage (Fig 3*F, J*), with expression of *Fz1* concentrating particularly in cells of the developing ORS (Fig 3*F*). *Fz10* is expressed at relatively high levels throughout the follicular epithelium and DP, and its expression is markedly elevated in hair follicles compared with interfollicular epidermis and dermis.

By the late peg and bulbous peg stages, specific expression of several additional *Fz* genes is initiated. Expression of *Fz5* appears in the developing ORS, particularly in the follicle bulb, at the bulbous peg stage (Fig 3*G*). Expression of *Fz6* is maintained in the epidermis throughout later embryonic development, and appears in central cells of the hair follicle epithelium at the bulbous peg stage (Fig 3*H*). Expression of *Fz7* is not elevated in the interfollicular epidermis, but specifically appears in central epithelial cells in the upper half of developing hair follicles at the late peg stage (Fig 3*D*), intensifying in these cells at the

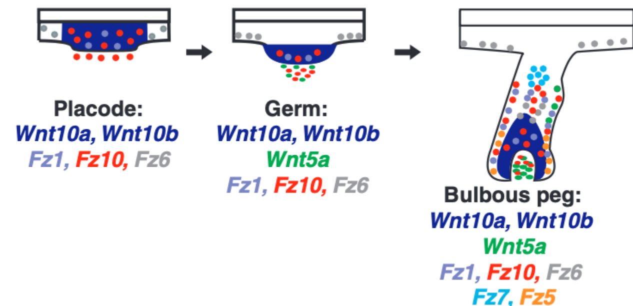

Figure 4
Summary of *Fz* expression in hair follicle morphogenesis and comparison with *Wnt* gene expression. Expression of *Wnt10a* and *Wnt10b* is represented by solid dark blue coloration. *Fz1* expression is represented by violet circles, *Fz10* by red circles, *Fz6* by gray circles, *Wnt5a* by green circles, *Fz5* by orange circles, and *Fz7* by light blue circles. *Wnt* gene expression data are taken from Reddy *et al* (2001).

right of the figure). *Wnt10b* and *Wnt10a* are expressed continuously in follicular epithelium during these later stages of morphogenesis. Canonical WNT signaling, however, assayed by TOPGAL expression, is downregulated in hair follicles at the germ to peg stages (DasGupta and Fuchs, 1999). It is therefore interesting to note that expression of *Wnt5a*, which is capable of antagonizing the WNT/β-catenin pathway (Weidinger and Moon, 2003), is

https://reader.elsevier.com/reader/sd/pii/S0022202X15309210?to…E8C0E7880A49CEE4891E7D7F20A8B51E65884FA010206F760B8E0E2A42049 Page 4 of 9

canonical signaling driven by WNT5A may predominate at these time points.

At the bulbous peg stage, *Wnt10a* and *Wnt10b* are strongly expressed in a cone of epithelial cells surrounding the DP (Reddy *et al*, 2001), and activation of canonical WNT signaling also appears to localize to these cells (DasGupta and Fuchs, 1999). Expression of *Fz6* and *Fz10* in cells adjacent to the DP is therefore consistent with activation of canonical WNT signaling by WNT10A/10B binding to FZ6 and FZ10 receptors. Further experiments will be required to determine whether *Wnt*-expressing and *Fz*-expressing WNT activated cells are intermingled at this stage, or whether the same cells express *Wnt* and *Fz* genes and activate the WNT pathway. The latter situation would be consistent with an autocrine as well as a paracrine mode of signaling. *Wnt5a* is strongly expressed, and *Wnt10a* is weakly expressed, in the DP at the bulbous peg stage (Reddy *et al*, 2001), consistent with the absence of canonical WNT reporter gene expression from the DP itself (DasGupta and Fuchs, 1999).

The expression patterns of *Wnt* and *Fz* genes at the various stages of follicle development were essentially identical between primary and secondary hair follicles and vibrissa follicles, indicating that differences in the size and growth properties of different types of hair follicle are not mediated by differential expression of these genes. In contrast, comparison of the expression patterns of *Wnt* genes in embryonic mouse skin and hair follicles with expression of the equivalent genes in embryonic chick skin and feather follicles reveals several striking differences, suggesting that divergence in the morphogenesis of hair and feather follicles might be achieved in part through differential expression of WNT family members (Reddy *et al*, 2001; Chodankar *et al*, 2003; Millar, 2003). Interestingly, *Fz1*, *Fz7*, and *Fz10* are expressed in developing feather follicles in chick embryos (Kawakami *et al*, 2000a; Chodankar *et al*, 2003) as well as in developing mouse hair follicles. More detailed analysis of *Fz* expression in chick skin and comparison with the results reported here may reveal additional mechanisms by which the diversity of appendage development is achieved.

***Fz* genes are expressed in specific subsets of cells in postnatal anagen follicles** To identify *Fz* genes expressed in mature anagen follicles, we used *in situ* hybridization of dorsal skin sections from mice at postnatal day 7 with probes for *Fz1*, *Fz2*, *Fz4*, *Fz5*, *Fz6*, *Fz7*, *Fz8*, *Fz9*, and *Fz10*. Of these, specific expression in hair follicles was not detected for *Fz2*, *Fz4*, *Fz8*, or *Fz9*. We found that *Fz1* and *Fz10* are expressed in the ORS, and in the DP (Fig 5*A, E*). *Fz5* is expressed predominantly in the ORS (Fig 5*B*), and *Fz6* and *Fz7* are expressed in precursor cells of the hair shaft cortex, with *Fz7* being expressed in mature precortical cells higher up in the follicle (Fig 5*D*), and *Fz6* in precortical cells that are exiting the proliferative matrix compartment and beginning the process of differentiation, as well as in adjacent IRS precursor cells (Fig 5*C*).

Expression of *Fz6* and *Fz7* in precursor cells of the hair

Two canonical *Wnt*s are expressed in cell layers immediately adjacent to the precortex: *Wnt3* is expressed in differentiating hair shaft medulla cells, whereas the related gene *Wnt3a* is expressed in differentiating IRS cells (Millar *et al*, 1999; Reddy *et al*, 2001) (Fig 5*F*, *middle diagram*). These expression patterns suggest that activation of the WNT/β-catenin pathway in precortical cells is mediated by interactions of WNT3 and WNT3A, secreted from the premedulla and IRS, with FZ6 expressed on early precortex cells, and FZ7 expressed in more differentiated precortical cells.

Expression of *Fz1*, *Fz5*, and *Fz10* in ORS cells is intriguing, as canonical WNT signaling has not been reported in this cell layer. However, DVL2, which is a component of both canonical and non-canonical WNT signaling pathways, is specifically expressed in a subset of ORS cells above the hair follicle bulb (Millar *et al*, 1999), and two *Wnt*s that generally activate non-canonical WNT signaling, *Wnt5a* and *Wnt11*, are expressed in, or immediately adjacent to, the ORS, specifically in this region (Reddy *et al*, 2001) (Fig 5*F*, *left diagram*). Thus, WNT5A and WNT11 may stimulate non-canonical WNT signaling via interactions with FZ1, FZ5, and/or FZ10. To date, non-canonical signaling activity has been demonstrated for FZ1 (Habas *et al*, 2001; Roman-Roman *et al*, 2004), but not FZ5 or FZ10, making FZ1 the strongest candidate for such a role. Possible functions for non-canonical WNT signaling in the ORS of anagen hair follicles include regulation of the movements of stem cell progeny from the bulge to the matrix (Oshima *et al*, 2001).

Expression of *Fz1* and *Fz10* is detected in the DP and matrix of anagen follicles, and several *Wnt*s are expressed in the follicle bulb (Fig 5*F*, *right diagram*), and could potentially interact with DP and matrix cells, in particular *Wnt10a*, *Wnt10b*, and *Wnt4* (Reddy *et al*, 2001). In addition, *Wnt5a* is weakly expressed in the DP itself (Reddy *et al*, 2001). Consistent with these data, expression of the BATgal WNT/β-catenin reporter has been demonstrated in the matrix and DP of hair follicles examined at postnatal day 4 (Maretto *et al*, 2003). Reporter gene activity was not detected in the DP of all follicles, suggesting that canonical WNT signaling in the DP may show temporal variability, depending on the precise stage of anagen examined. Such variability might be regulated in part by the balance of canonical and non-canonical WNTs expressed in the follicle bulb.

***Fz* genes are expressed in the secondary germ at anagen onset** To determine the patterns of *Fz* gene expression at telogen and anagen onset, 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. *Fz2*, *Fz4*, *Fz5*, *Fz8*, and *Fz9* were not specifically expressed in the skin or hair follicles of P23 skin. *Fz1*, *Fz6*, *Fz7*, and *Fz10* showed low levels of expression in the epithelium of telogen hair follicles (Fig 6*A*, *C*, *E*, *G*). At anagen onset, all of these

Expression of Frizzled Genes in Developing and Postnatal Hair Follicles | Elsevier Enhanced Reader 9/24/19, 1:20 PM

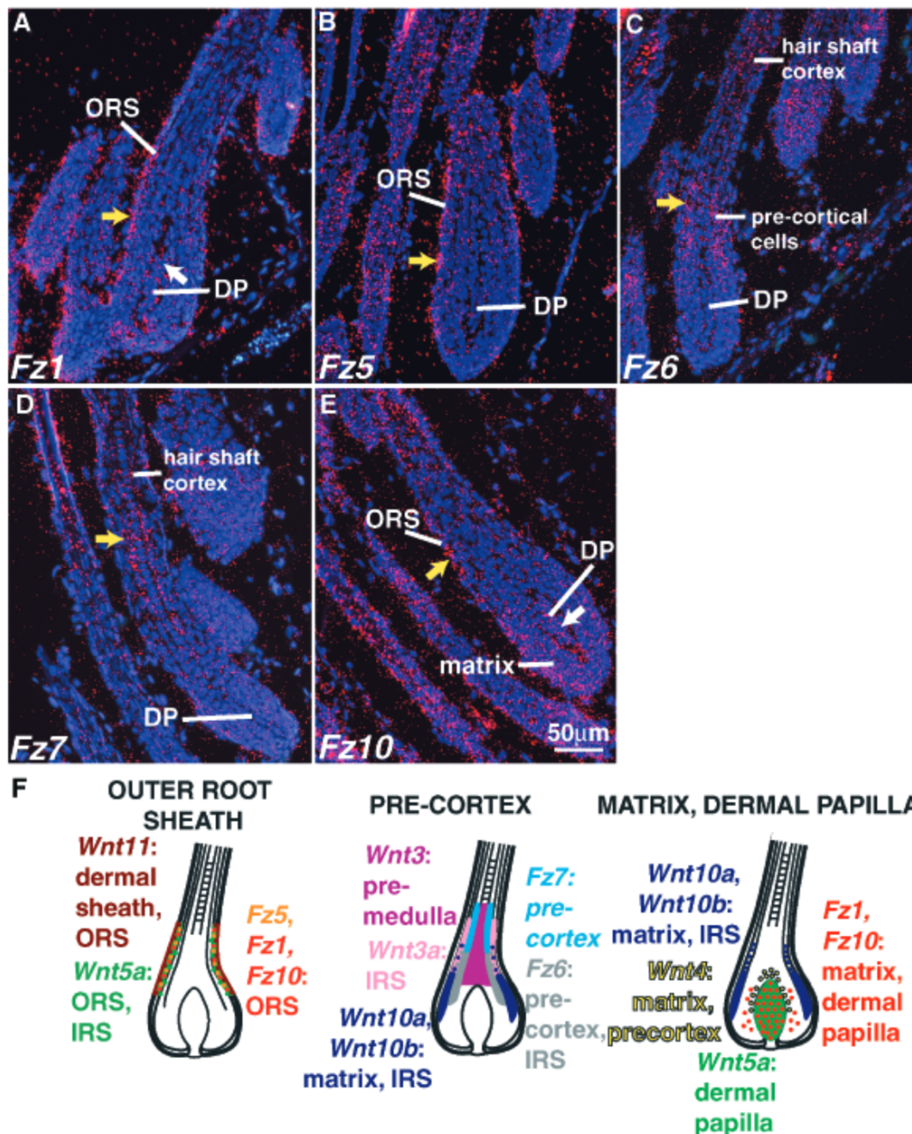

Figure 5
Expression of *Fz* genes during anagen. Sections of dorsal skin at postnatal day 7 were hybridized with probes for *Fz1* (*A*), *Fz5* (*B*), *Fz6* (*C*), *Fz7* (*D*), and *Fz10* (*E*). (*A*) *Fz1* is expressed in a subset of ORS cells above the follicle bulb (*yellow arrow*) and, at lower levels, in the DP (*white arrow*). (*B*) *Fz5* is expressed in the ORS (*yellow arrow*). (*C*) *Fz6* is expressed in precursor cells of the hair shaft cortex (*arrow*), as they exit the proliferative matrix compartment in the follicle bulb, and in adjacent IRS precursor cells. (*D*) *Fz7* is expressed in more differentiated precursors of the hair shaft cortex, higher up in the follicle (*arrow*). (*E*) *Fz10* is expressed in the ORS (*yellow arrow*), in matrix cells, and in the DP (*white arrow*). (*F*) Summary diagrams of *Fz* expression in anagen hair follicles and comparison with the expression of *Wnt* genes (Reddy *et al*, 2001). Major sites of *Fz* gene expression (the ORS; precursor cells of the hair shaft cortex; and the matrix and DP) are indicated above each diagram in which the domains of relevant *Fz* gene expression, and adjacent *Wnt* expression are indicated. *Fz* gene expression is represented by the following colors: *Fz1*, *Fz10*, red; *Fz5*, orange; *Fz6*, gray; *Fz7*, pale blue. *Wnt* gene expression is indicated by the following colors: *Wnt11*, brown; *Wnt5a*, green; *Wnt3*, dark pink; *Wnt3a*, pale pink; *Wnt10a* and *Wnt10b*, dark blue; *Wnt4*, yellow. The photographs in panels (*A–E*) were taken at the same magnification. *Scale bar* = 25 μm (panel *E*). ORS, outer root sheath; DP, dermal papilla; IRS, inner root sheath.

expressed at anagen onset, making it unlikely that this process is driven by regulated expression of *Fz*. Instead, expression of *Fz* genes at low levels during telogen and association of *Fz* expression with the proliferating secondary hair germ in very early anagen indicates that hair follicle cells are capable of receiving WNT signals at these stages and could potentially respond to *Wnt*s *10a* and *10b* that are upregulated at anagen onset (Reddy *et al*, 2001).

In summary, here we show that *Fz* genes are expressed in complex patterns during hair follicle morphogenesis and postnatal hair growth. *Fz* gene expression patterns exhibit extensive overlap, with two or more *Fz* genes often being expressed at the same site. In addition to expression of the *Fz* genes described here, *Fz3* shows transient expression in hair follicle placodes at E15 (Hung *et al*, 2001), overlapping with the expression of *Fz1* and *Fz10*. In postnatal hair skin, and that analysis of the effects of loss of *Fz* function is likely to require the generation of mice bearing multiple mutations.

At each developmental stage, the pattern of *Fz* expression in developing and postnatal hair follicles can be clearly correlated with the expression of genes encoding WNT ligands, allowing us to identify candidate receptors for each of the WNTs. Specific *Fz* genes are expressed at each site where activity of the canonical WNT signaling pathway has been described. In addition, *Fz* genes are expressed at sites in the skin where the canonical pathway is not active, but that overlap with, or are adjacent to, cell populations displaying expression of non-canonical WNT ligands. These sites include the interfollicular epidermis, and specific subpopulations of cells in hair follicles. It will be interesting in the future to determine whether the distribution of WNT

https://reader.elsevier.com/reader/sd/pii/S0022202X15309210?to…E8C0E7880A49CEE4891E7D7F20A8B51E65884FA010206F760B8E0E2A42049 Page 6 of 9

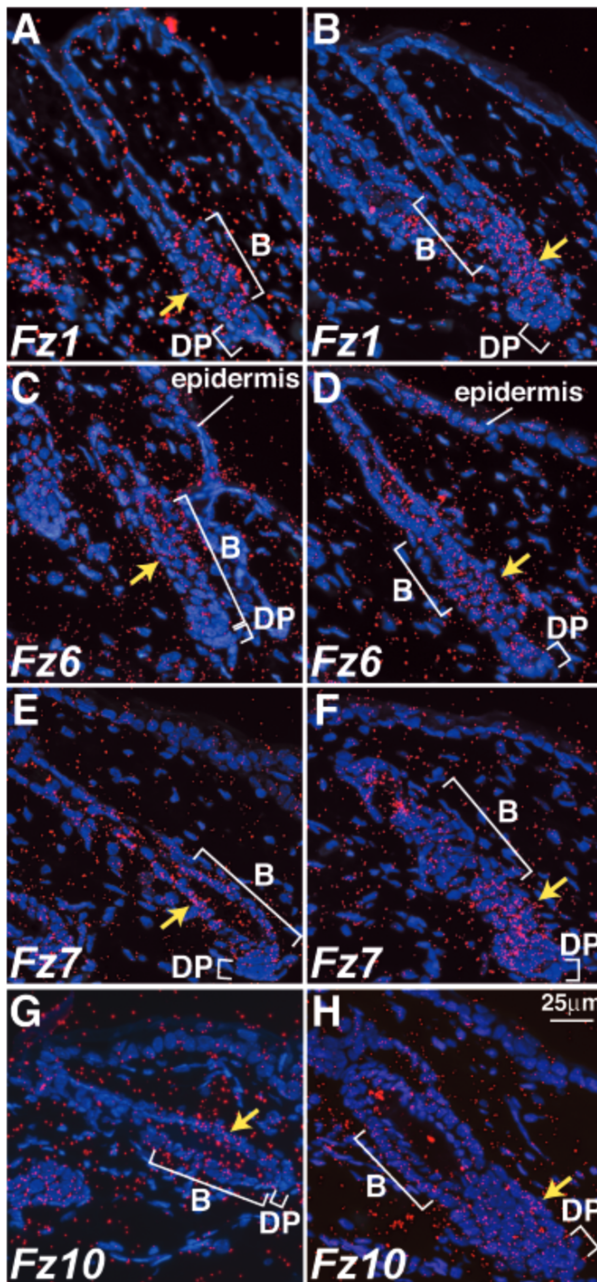

Figure 6
Expression of *Fz* genes in telogen and at anagen onset. Sections of dorsal skin at postnatal day 23 were probed with *Fz1* (*A, B*), *Fz6* (*C, D*), *Fz7* (*E, F*), and *Fz10* (*G, H*). Telogen stage follicles are shown in panels (*A, C, E, G*); follicles entering anagen are shown in panels (*B, D, F, H*). Hybridization is indicated by arrows. All four *Fz* genes are expressed at low levels in the epithelium of telogen follicles (*A, C, E, G*). *Fz6* is also expressed in the epidermis (*C, D*). All four *Fz* genes are expressed in the secondary germ at anagen onset (*B, D, F, H*) (*arrows*). *Scale bar* = 25 μm (panel *H*). DP, dermal papilla; B, bulge.

nature of hair follicles, the known roles of non-canonical WNT signaling in the regulation of cell movements in vertebrates (Habas *et al*, 2003; Takeuchi *et al*, 2003; Ulrich *et al*, 2003), and recent data indicating antagonism between

Materials and Methods

Mice and tissues All studies involving animals were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania School of Medicine. BALB/c postnatal mice and FVB/N embryos from timed natural matings were used as the sources of tissue.

RT-PCR For RT-PCR experiments, we dissected dorsal skin from FVB/N mouse embryos at embryonic day 14.5 (E14.5) and extracted RNA using Trizol (Gibco BRL, Rockville, Maryland). RNA preparations were treated with DNAse I (Roche-Boehringer-Mannheim, Indianapolis, Indiana) to remove any contaminating genomic DNA, and were purified using Rneasy columns (Qiagen, Valencia, California). cDNA was synthesized using 5 μg of RNA in a volume of 40 μL. Two microliters of each reverse transcription (RT) reaction was subjected to 30 rounds of PCR using specific primers for mouse *Fz* 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 primers were used to amplify the following sequences whose Genbank accession numbers are shown in brackets: *Fz1*, 1522–1857 (af054623); *Fz2*, 41–311 (af139183); *Fz3*, 1967–2373 (u43205); *Fz4*, 1491–1964 (u43317); *Fz5*, 1502–1771 (xm_192878); *Fz6*, 1864–2271 (u43319); *Fz7*, 1791–2193 (u43320); *Fz8*, 1791–2066 (U43321); and *Fz9*, 1323–1759 (AF088850).

***In situ* hybridization** Anterior-posterior strips of skin were dissected from the mid-dorsum of postnatal mice 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 easier penetration of fixative. Fixation was carried out overnight in 4% paraformaldehyde at 4°C prior to dehydration and embedding. Embryos were sectioned sagittaly, and dorsal skin strips were sectioned parallel to the anterior–posterior axis. ^{35}S-labeled antisense riboprobes for *Fz2* and *Fz4* were prepared from linearized pBluescript plasmid subclones using either T3 or T7 RNA polymerase as described previously (Wang *et al*, 1996). Probe templates for *Fz1*, *Fz5*, *Fz6*, *Fz7*, *Fz8*, and *Fz9* were synthesized by PCR of mouse embryo cDNA using the primers described above. Probe template for *Fz10* was synthesized by PCR of mouse embryo cDNA using 18 base pair primers to amplify nucleotides 1501–2030 of *Fz* cDNA (accession number af206321). Sequences for the binding of T7 RNA polymerase were added to the 3′ primers to create templates for the synthesis of antisense probes and to the 5′ primers for creating sense probe templates. *In situ* hybridization was carried out as described previously (Reddy *et al*, 2001). Sections were counterstained with 2 μg/mL Hoechst 33258 dye (Sigma, St Louis, Missouri), mounted in 50% Canada balsam in methyl salicylate (Sigma), and were photographed using an Olympus Bx60 microscope with an MVI Darklite stage adaptor and Photometrics Coolsnap digital camera.

We thank Jeremy Nathans for *Fz* plasmid probe templates, and George Cotsarelis for comments on the manuscript.

DOI: 10.1111/j.0022-202X.2004.23215.x

Manuscript received January 29, 2004; revised March 19, 2004; accepted for publication March 29, 2004

Address correspondence to: Sarah E. Millar, PhD, M8D Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia, PA 19104-6100, USA. Email: millars@mail.med.upenn.edu

Expression of Frizzled Genes in Developing and Postnatal Hair Follicles | Elsevier Enhanced Reader 9/24/19, 1:20 PM

Axelrod JD, Miller JR, Shulman JM, Moon RT, Perrimon N: Differential recruitment of dishevelled provides signaling specificity in the planar cell polarity and wingless signaling pathways. Genes Dev 12:2610–2622, 1998

Chodankar R, Chang CH, Yue Z, *et al*: Shift of localized growth zones contributes to skin appendage morphogenesis: Role of the Wnt/beta-catenin pathway. J Invest Dermatol 120:20–26, 2003

Cotsarelis G, Sun TT, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329–1337, 1990

DasGupta R, Fuchs E: Multiple roles for activated Lef/Tcf transcription complexes during hair follicle development and differentiation. Development 126: 4557–4568, 1999

Dry FW: The coat of the mouse (*Mus musculus*). J Genet 16:287–340, 1926

Habas R, Dawid IB, He X: Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. Genes Dev 17:295–309, 2003

Habas R, Kato Y, He X: Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel formin homology protein Daam1. Cell 107:843–854, 2001

Hardy MH: The secret life of the hair follicle. Trends Genet 8:55–60, 1992

He X, Saint JJ, Wang Y, Nathans J, Dawid I, Varmus H: A member of the Frizzled protein family mediating axis induction by wnt-5a. Science 275:1652–1654, 1997

Huelsken J, Birchmeier W: New aspects of Wnt signaling pathways in higher vertebrates. Curr Opin Genet Dev 11:547–553, 2001

Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W: Beta-catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105:533–545, 2001

Hung BS, Wang XQ, Cam GR, Rothnagel JA: Characterization of mouse Frizzled-3 expression in hair follicle development and identification of the human homolog in keratinocytes. J Invest Dermatol 116:940–946, 2001

Kawakami Y, Wada N, Nishimatsu S, Komaguchi C, Noji S, Nohno T: Identification of chick Frizzled-10 expressed in the developing limb and the central nervous system. Mech Dev 91:375–378, 2000a

Kawakami Y, Wada N, Nishimatsu S, Nohno T: Involvement of Frizzled-10 in Wnt-7a signaling during chick limb development. Dev Growth Differ 42: 561–569, 2000b

Kawano Y, Kypta R: Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116:2627–2634, 2003

Korinek V, Barker N, Willert K, *et al*: Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. Mol Cell Biol 18:1248–1256, 1998

Kuhl M, Sheldahl LC, Malbon CC, Moon RT: Ca(2 +)/calmodulin-dependent protein kinase Ii is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. J Biol Chem 275:12701–12711, 2000

Lin MH, Leimeister C, Gessler M, Kopan R: Activation of the notch pathway in the hair cortex leads to aberrant differentiation of the adjacent hair-shaft layers. Development 127:2421–2432, 2000

Malbon CC, Wang H, Moon RT: Wnt signaling and heterotrimeric G-proteins: Strange bedfellows or a classic romance? Biochem Biophys Res Commun 287:589–593, 2001

Maretto S, Cordenonsi M, Dupont S, *et al*: Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci USA 100:3299–3304, 2003

Millar SE: Molecular mechanisms regulating hair follicle development. J Invest Dermatol 118:216–225, 2002

Millar SE: Wnts: Multiple genes, multiple functions. J Invest Dermatol 120:7–8, 2003

Millar SE, Willert K, Salinas PC, Roelink H, Nusse R, Sussman DJ, Barsh GS: Wnt signaling in the control of hair growth and structure. Dev Biol 207: 133–149, 1999

Moon RT, Bowerman B, Boutros M, Perrimon N: The promise and perils of Wnt signaling through beta-catenin. Science 296:1644–1646, 2002

Muller-Rover S, Handjiski B, van der Veen C, *et al*: A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol 117:3–15, 2001

Oliver RF, Jahoda CA: Dermal–epidermal interactions. Clin Dermatol 6:74–82, 1988

Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y: Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104: 233–245, 2001

Pandur P, Lasche M, Eisenberg LM, Kuhl M: Wnt-11 Activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418: 636–641, 2002

Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J: A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of Frizzled receptors. Proc Natl Acad Sci USA 94:2859–2863, 1997

Reddy S, Andl T, Bagasra A, Lu MM, Epstein DJ, Morrisey EE, Millar SE: 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. Mech Dev 107:69–82, 2001

Roman-Roman S, Shi DL, Stiot V, *et al*: Murine Frizzled-1 behaves as an antagonist of the canonical Wnt/beta-catenin signaling. J Biol Chem 279:5725–5733, 2004

Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA: Inhibition of adipogenesis by Wnt signaling. Science 289:950–953, 2000

Sheldahl L, Park M, Malbon C, Moon R: Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Curr Biol 9:695–698, 1999

Slusarski DC, Yang-Snyder J, Busa WB, Moon RT: Modulation of embryonic intracellular Ca^{2+} Signaling by Wnt-5a. Dev Biol 182:114–120, 1997

Sperling LC: Hair anatomy for the clinician. J Am Acad Dermatol 25:1–17, 1991

Sumanas S, Strege P, Heasman J, Ekker SC: The putative Wnt receptor Xenopus Frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. Development 127:1981–1990, 2000

Takeuchi M, Nakabayashi J, Sakaguchi T, Yamamoto TS, Takahashi H, Takeda H, Ueno N: The prickle-related gene in vertebrates is essential for gastrulation cell movements. Curr Biol 13:674–679, 2003

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM: Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102:451–461, 2000

Terasaki H, Saitoh T, Shiokawa K, Katoh M: Frizzled-10, up-regulated in primary colorectal cancer, is a positive regulator of the Wnt–beta-catenin–Tcf signaling pathway. Int J Mol Med 9:107–112, 2002

Topczewski J, Sepich DS, Myers DC, *et al*: The zebrafish *Glypican knypek* controls cell polarity during gastrulation movements of convergent extension. Dev Cell 1:251–264, 2001

Ulrich F, Concha ML, Heid PJ, *et al*: Slb/Wnt11 controls hypoblast cell migration and morphogenesis at the onset of zebrafish gastrulation. Development 130:5375–5384, 2003

van Genderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, Bruhn L, Grosschedl R: Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in Lef-1-deficient mice. Genes Dev 8:2691–2703, 1994

Veeman MT, Axelrod JD, Moon RT: A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Developmental Cell 5:367–377, 2003

Wang J, Shackleford GM: Murine Wnt10a and Wnt10b: Cloning and expression in developing limbs, face and skin of embryos and in adults. Oncogene 13:1537–1544, 1996

Wang Y, Macke JP, Abella BS, *et al*: A large family of putative transmembrane receptors homologous to the product of the drosophila tissue polarity gene Frizzled. J Biol Chem 271:4468–4476, 1996

Weidinger G, Moon RT: When Wnts antagonize Wnts. J Cell Biol 162:753–755, 2003

Westfall TA, Brimeyer R, Twedt J, Gladon J, Olberding A, Furutani-Seiki M, Slusarski DC: Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/beta-catenin activity. J Cell Biol 162:889–898, 2003

Wong GT, Gavin BJ, McMahon AP: Differential transformation of mammary epithelial cells by Wnt genes. Mol Cell Biol 14:6278–6286, 1994

Yang-Snyder J, Miller JR, Brown JD, Lai CJ, Moon RT: A Frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr Biol 6:1302–1306, 1996

Zorn AM: Wnt signalling: Antagonistic dickkopfs. Curr Biol 11:R592–R595, 2001

https://reader.elsevier.com/reader/sd/pii/S0022202X15309210?to…E8C0E7880A49CEE4891E7D7F20A8B51E65884FA010206F760B8E0E2A42049 Page 8 of 9