Dermal β-catenin activity in response to epidermal Wnt ligands is required for fibroblast proliferation and hair follicle initiation

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SUMMARY
Dermal fibroblasts are required for structural integrity of the skin and for hair follicle development. Uniform Wnt signaling activity is present in dermal fibroblast precursors preceding hair follicle initiation, but the functional requirement of dermal Wnt signaling at early stages of skin differentiation and patterning remains largely uncharacterized. We show in mice that epidermal Wnt ligands are required for uniform dermal Wnt signaling/β-catenin activity and regulate fibroblast cell proliferation and initiation of hair follicle placodes. In the absence of dermal Wnt signaling/β-catenin activity, patterned upregulation of epidermal β-catenin activity and Edar expression are absent. Conversely, forced activation of β-catenin signaling leads to the formation of thickened dermis, enlarged epidermal placodes and dermal condensates that result in prematurely differentiated enlarged hair follicles. These data reveal functional roles for dermal Wnt signaling/β-catenin in fibroblast proliferation and in the epidermal hair follicle initiation program.

KEY WORDS: Dermis, Mouse embryo, Fibroblasts, Hair follicle, Wnt, β-catenin

INTRODUCTION
The dermis contributes to the structural integrity and function of skin. Dermal fibroblasts in the dermis are crucial for hair follicle formation and wound healing (Millar, 2002). Dermal fibroblasts orchestrate the physiology of the skin by communicating with other resident cell types, including epidermal keratinocytes, vascular endothelial cells and neurons (Ansel et al., 1996; Detmar, 1996; Werner and Smola, 2001). Mammalian dermal fibroblasts are derived from multiple embryonic tissues: the head dermis derives from neural crest and paraxial mesoderm, the dorsal dermis originates from somitic mesoderm, and the ventral dermis arises from lateral plate mesoderm (Atit et al., 2006; Ohtola et al., 2008; Tran et al., 2010; Yoshida et al., 2008). Between E10.5 and E12.5, loose mesenchyme cells migrate to the subectodermal region and become specified to dermal fibroblast precursors (Atit et al., 2006; Dhouailly et al., 2004; Ohtola et al., 2008; Tran et al., 2010). The dermal fibroblast precursors then differentiate into matrix-secreting dermal fibroblasts or dermal condensates, dermal papillae and dermal sheath cells that are induced by the developing hair follicle (Dhouailly et al., 2004; Reynolds and Jahoda, 1991).

Hair follicle development is initiated at E13.5 in the epidermis and requires input from dermal fibroblast progenitors (Millar, 2002). Heterotransplant assays suggest that the dermis provides the first signal to initiate the formation of patterned epidermal thickenings that will become hair follicle placodes (Hardy, 1992). However, the exact nature of this ‘first dermal signal’ and whether this signal is uniform or patterned are unknown. Between E13.5 and E14.5 in the mouse embryo, reciprocal signaling from the placodes leads to aggregation of underlying dermal fibroblast progenitors into dermal condensates (Hardy, 1992). Dermal condensates/dermal papillae provide the necessary signals for proliferation, downgrowth and regeneration of hair follicular epidermal cells (Horne et al., 1986; Jahoda et al., 1984; Jahoda et al., 1993).

Canonical Wnt/β-catenin signaling may have early and diverse roles in hair follicle-associated and interfollicular dermal fibroblasts. Wnt ligands are lipid-modified extracellular glycoproteins that require the activity of wntless (Evi or GPR177) protein (Wls) for secretion (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006; Grigoryan et al., 2008). Extracellular Wnt proteins bind to Frizzled receptors and low-density related lipoproteins (LRPs) in the target cell membrane and stabilize the intracellular transducer β-catenin, which then translocates into the nucleus and binds to TCF/Lef family transcription factors to activate the expression of cell type-specific target genes (van Amerongen and Nusse, 2009). The dorsal neural tube provides a signal, which can be substituted by Wnt1, to induce the specification of dermal progenitors from the medial dermomyotome in the chick embryo (Olivera-Martinez et al., 2001). Several canonical Wnt ligands, such as Wnt1, Wnt3a, Wnt4 and Wnt6, are expressed uniformly throughout dorsal surface epithelium, preceding feather/hair placode formation, in chick and mouse embryos (Andl et al., 2002; Chang et al., 2004; DasGupta and Fuchs, 1999; Reddy et al., 2001; Rodriguez-Niedenfuhr et al., 2003; Wagner et al., 2000). At E14.5, the second wave of epidermal Wnt ligand expression of Wnt10b, Wnt10a, Wnt7a and Wnt7b occurs as patterned upregulation in hair follicle placodes (Reddy et al., 2001). Wnt5a, Wnt10a and Wnt11 mRNAs are expressed in the upper dermis or dermal condensate at E14.5 (Reddy et al., 2001). The secreted Wnt inhibitors Dkk1 and Dkk4 are expressed in the dermis surrounding the dorsal condensate and in the epidermis hair follicle preplacode, respectively, at E14.5 (Andl et al., 2002; Bazzi et al., 2007). Overexpression of Dkk1 in the epidermis leads to complete lack of hair follicle formation (Andl et al., 2002), whereas overexpression of Dkk4 causes malformed secondary hair follicles without affecting the primary follicles (Cui et al., 2010). Canonical
Wnt signaling reporter mice show activity throughout the upper dermis, four to six cell layers below the epidermis, from E11.5 to E15.5. From E13.5 onwards, elevated Wnt reporter expression can be seen in hair follicle placodes and in the hair follicle-associated dermal condensates (DasGupta and Fuchs, 1999; Zhang et al., 2009). However, the source of the Wnt ligands for dermal Wnt signaling/β-catenin activity remains to be defined by tissue-restricted and cell type-specific deletion of all Wnt ligands. It has been hypothesized that dermal Wnt signaling/β-catenin activity is the initiation cue for hair follicle development (Millar, 2002; Schmidt-Ullrich and Paus, 2005). In the absence of appropriate embryonic dermal fibroblast-specific spatiotemporal genetic tools, the function of dermal Wnt signaling/β-catenin activity in fibroblast differentiation and hair follicle initiation remains to be identified.

To address these questions, we utilized tissue-specific genetic manipulation of Wls and β-catenin (Ctnnb1) activity preceding hair follicle initiation at E13.5. We show that after fibroblast cell fate selection, dermal β-catenin activity levels regulate the proliferation of embryonic fibroblast progenitors and dermal thickness. We also find that uniform dermal β-catenin activity in the upper dermis is in response to epidermal Wnt ligands and is required upstream of all the earliest known events in hair follicle placode initiation. Our data reveal that dermal Wnt signaling/β-catenin activity is necessary for hair follicle initiation.

MATERIALS AND METHODS

Generation and genotyping of mouse lines
En1Cre (Kimmel et al., 2000), HoxB6CreERT2 (Nguyen et al., 2009), K14Cre (Dassule et al., 2000), ROSA26R (Soriano, 1999), Wntlesslox (Carpenter et al., 2010), β-cateninfllox (Brauhl et al., 2001), β-cateninfllox (Harada et al., 1999) and TCF/Lef-lacZ (Liu et al., 2003) were genotyped from ear punch biopsy as previously reported. To stabilize β-catenin in ventral dermal progenitors, tamoxifen dissolved in corn oil (20 mg/ml, K14CreIACUC committee. Controls from one to three litters were analyzed. All animal procedures (2008). For each experiment, at least three to five mutants with littermate pairs were genotyped

RESULTS

Canonical Wnt signaling reporter is active in the dermis and Wls is broadly expressed in the epidermis prior to hair follicle initiation

We used TCF/Lef-lacZ reporter mice (Liu et al., 2003) to visualize the spatiotemporal activation of canonical Wnt signaling/β-catenin in the dermal fibroblasts (Fig. 1A-C). Similar to Conductin-lacZ (Conductin is also known as Axin2) reporter mice (Zhang et al., 2008). RNA extraction was performed using Trizol (Invitrogen). cDNA from total RNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (ABI). For quantitative PCR, TaqMan primers (ABI, Mm00509695_m1) were used following the manufacturer’s instructions. Relative mRNA expression levels were calculated based on the ΔΔCt method.

Cell survival, proliferation, quantification and statistical analysis

Cell survival was assayed by TUNEL staining using the TMR-Red Kit according to the manufacturer’s instructions (Roche). Cryosections of embryos were used for proliferation analysis (n=3-6 embryos, 12-20 fields at 40× magnification for each genotype). A fixed area of the upper dermis (190 μm length × 25 μm height directly beneath epidermis) was used for calculating cell density and proliferation index, as previously described (Ohtola et al., 2008). For placode cell number and proliferation index of the placode and dermal condensate, E14.5 skin was cryosectioned at 7-10 μm and processed for Dkk4 and Lef1 mRNA in situ hybridization and BrdU immunohistochemistry. The number of pixels covering the hair follicle invaginated into the dermis was measured from photographs of follicles at 40× magnification in the ventral skin at E15.5-17.5 (n=5 embryos) using ImageJ software (NIH). For quantification of hair follicle number and stage distribution across different time points, 30 low-magnification fields of ventral skin sections were examined. Hair follicles were staged as previously described (Paus et al., 1999). Statistical significance was calculated using Student’s t-test.

Histology, in situ hybridization, immunohistochemistry and quantitative PCR

Whole-mount and section X-Gal staining, whole-mount and section in situ hybridization and immunohistochemistry were performed as described previously (Andl et al., 2002; Atit et al., 2006; Ohtola et al., 2008; Rivera-Perez et al., 1999). In situ probes were: Axin2 from Addgene; Wnt10b from Mayumi Ito (New York University); Twist2 (Dermo1) from Eric Olsen (University of Texas Southwestern Medical Center); Wnt11 from Steve Potter (Cincinnati Children’s Hospital Medical Center); Egr1 from Marja Mikkola (University of Helsinki); collagen type I (Col1a1) from Mayumi Ito (New York University); and Lef1 from Fanxin Long (Washington University in St Louis). In situ probes for bone morphogenetic protein 4 (Bmp4), Bmp7, patched 1 (Ptc1), sonic hedgehog (Shh) and fibroblast growth factor receptor 1 (Fgfr1) were from Gail Martin (University of California, San Francisco). Antisense probe for Dkk4 was generated by PCR using forward primer GCTTATTTAGGTGACACTATAG and reverse primer TTTGTAATACGACTCACTATAGGGAGGGCT-ACACAGTGAGAGCC.

For immunohistochemistry, primary antibodies against α-smooth muscle actin (α-SMA; Dako), Cre (Novagen), filaggrin (Covance), keratin 10 (Covance), keratin 14 (Covance), keratin 17 (Abcam), Sox9 (Chemicon), Sox2 (Santa Cruz), Lef1 (Cell Signaling Technology), PGP9.5 (Ultraclone) and Pecam1 (BD), and AE13 antibody (Abcam) were used. For detection of alkaline phosphatase activity, sections were fixed in acetone for 20 minutes on ice, rinsed twice with PBS, and then incubated with the NBT/BCIP (Roche) substrate.

Fig. 1. Wnt signaling reporter activity and Wls protein expression in embryonic mouse skin. (A-C) X-Gal staining of transverse sections of dorsal skin at the forelimb level from Wnt reporter TCF-Lef-lacZ embryos. (D-G) Wls protein expression in embryonic dorsal skin revealed detectable expression in the epidermis, hair follicle placode, hair follicle peg (peg) and dermal condensate (dc). Dashed lines indicate the dermal-epidermal boundary. Dotted lines indicate the dermal condensate boundary.
Wnt signaling activity was sustained in the upper dermal progenitors between E12.5 and E14.5 in the TCF/Lef-lacZ reporter mice (Zhang et al., 2009). At E14.5, Wnt reporter expression was prominent in the hair follicle placode and in the underlying dermal condensate (Fig. 1C).

We examined expression of Wls protein by immunohistochemical staining in dorsal skin between E12.5 and E15.5 during primary (guard) hair follicle initiation. Wls protein was detectable in the surface ectoderm between E12.5 and E15.5 as punctate cytoplasmic staining and was enriched in the early developing hair follicle placode (Fig. 1D-G). In the dermal layer, Wls protein expression was detectable in the early dermal condensate starting at E14.5 (supplementary material Fig. S4A) (Reddy et al., 2001), but Wls protein expression was barely detectable by immunohistochemistry in the remaining upper dermis from E12.5-15.5 (Fig. 1D-G).

**Sustained β-catenin activity leads to an increase in dermal thickness and to proliferation of dermal fibroblasts**

It is not known whether activation of the Wnt signaling/β-catenin pathway in vivo is sufficient to promote the dermal fibroblast lineage. Owing to the embryonic lethality by E12.5 of mutants with sustained β-catenin activity generated with the En1Cre line (Atit et al., 2006; Ohtola et al., 2008), we generated HoxB6Cre-ER\textsuperscript{T1}/+; R26R/+; β-catenin\textsuperscript{loxP/loxP} mutant embryos (Brault et al., 2001; Harada et al., 1999; Nguyen et al., 2009), in which a stabilized form of β-catenin protein was induced in ventral dermal fibroblast precursors between E10.5 and E12.5 (Fig. 2A-H; supplementary material Fig. S1A,B, Fig. S2A-F). Wnt reporter activity and expression of the Wnt target gene Axin2 (Jho et al., 2002) were restricted to the upper dermis of the control embryo, whereas an expanded domain of expression was observed in the lineage-labeled cells in HoxB6Cre-ER\textsuperscript{T1}/+; R26R/+; β-catenin\textsuperscript{loxP/loxP} mutant embryos at E13.5 (supplementary material Fig. S1C-F).

Histological analysis of ventral skin from E13.5-17.5 revealed a thickened dermal layer in the mutant, with predominantly lineage-marked β-gal\textsuperscript{+} cells between the epidermis and sternum with enlarged hair follicles (Fig. 2A-H; supplementary material Fig. S2A-F). At E14.5-15.5, Fgfr1 and Twist2 (Li et al., 1995) were expressed in the upper dermis in control embryos, but had expanded domains in the conditional β-catenin\textsuperscript{loxP/loxP} mutant embryos (Fig. 2I-L). At E17.5, Col1a1 mRNA was detectable in the ventral dermis of control embryos and in the thickened dermis of activated β-catenin mutant embryos (Fig. 2M,N). We found a significant increase in the proliferation index of dermal fibroblasts in the upper dermis (Fig. 2P) in the activated β-catenin mutant embryos compared with the control littermates at E14.5. Moreover, there was a significant increase in cell density in the upper dermis of mutant embryos compared with controls between E13.5 and E15.5 (Fig. 2Q). Spatiotemporal activation of β-catenin activity in the dermis was therefore sufficient to lead to an expanded domain of canonical Wnt signaling-responsive fibroblast cells that proliferate more than normal and contribute to a thickened dermis.

**Sustained β-catenin activity in the developing dermis leads to larger hair follicle placodes and accelerated differentiation of hair follicles**

In our analysis of activated mutant dermis, we found enlarged hair follicles in regions of thickened dermis (Fig. 2C-H; supplementary material Fig. S2A-F). We examined whether
sustained activity of dermal β-catenin in the dermis alters hair follicle patterning and differentiation. First, we performed whole-mount in situ hybridization for mRNA expression of the hair follicle preplacode marker Dkk4 (Bazzi et al., 2007) in the HoxB6Cre-ER<sup>T1/+</sup>; R26R/+; β-catenin<sup>ex3/+</sup> mutant embryos at E14.5 and whole-mount X-Gal staining for Wnt signaling reporter activity in HoxB6Cre-ER<sup>T1/+</sup>; TCF/Lef-lacZ/+; β-catenin<sup>ex3/+</sup> mutant embryos at E15.5. We found an increase in both the size and number of hair follicle placodes that led to a perturbation of the evenly spaced hexagonal pattern and a decrease in interfollicular distance (Fig. 3A-D). Next, we performed section in situ hybridization for Dkk4 and Lef1 (DasGupta and Fuchs, 1999; Kratochwil et al., 1996) to quantify the cell number in hair follicle placode and dermal condensate, respectively (Fig. 4C,D, see Fig. 6G). The numbers of Dkk4-expressing epidermal placode cells and Lef1-expressing dermal condensate cells were higher in conditional β-catenin<sup>ex3/+</sup> mutant embryos than in the controls (Fig. 3E,F). However, the

Fig. 3. Increase in hair follicle placode size and number and accelerated differentiation in stabilized dermal β-catenin embryos. (A-D) Whole-mount in situ hybridization for Dkk4 at E14.5 (A,B) and X-Gal staining of Wnt reporter activity in mouse ventral skin (C,D). Expanded placodes in mutant embryos are indicated by arrows (B); the distance between placodes was decreased in the mutant skin (D, solid black lines). (E,F) Increase in the number of cells in the Dkk4<sup>+</sup> and Lef1<sup>+</sup> domains in mutant embryos as compared with the control. (G,H) Quantification of hair follicle area showed enlargement of hair follicle in mutant compared with control skin of age-matched littermate and enlargement of mutant hair follicle compared with stage-matched wild-type hair follicle. (I-L) K17 and Sox9 showed comparable expression in the outer root sheath (ORS) in control and mutant skin. (M,N) AE13 immunoreactivity was absent in the control ventral skin, but was present in the enlarged follicles of the mutant skin at E17.5. (O,P) Sox2 was present in the dermal condensate and papilla of the control and enlarged mutant hair follicles (arrows). Numbers (1-4) specify developmental stages of hair follicles. Dashed line demarcates the dermal-epidermal boundary. Control and mutant skin were photographed at the same magnification. Error bars indicate s.d.
increased size of epidermal placodes was not due to an increase in the proliferation index in mutants at E14.5, as judged by BrdU incorporation (51.04±8.93% in mutant versus 49.27±9.01% in control; n=30 placodes from 4-6 embryos). Moreover, we found that the proliferation index of the enlarged dermal condensate was slightly lower in the mutants (22.36±9.87% versus 30.94±12.54% in control). Thus, the sustained activation of β-catenin activity in the developing dermis either indirectly or directly promotes enlargement of dermal condensate and hair follicle placodes.

To determine the progression of the phenotype, we first characterized the stage distribution of hair follicles at different time points between E13.5 and E17.5. No hair follicle placode formation was detectable in the control or mutant ventral skin at E13.5 (Fig. 2A,B; supplementary material Fig. S1C,D; Table 1). At E14.5, there was an increase of stage 1 hair follicles but no premature progression of hair follicle placode differentiation in the mutant embryos compared with controls (Fig. 2C,D; supplementary material Fig. S2A,B; Table 1). By E15.5, the control had predominantly stage 1 and 2 hair follicles, whereas mutants had a greater number of follicles in stage 1 and 2 and some had progressed to stage 3 and 4 (Fig. 2E,F; supplementary material Fig. S2C,D; Table 1). Similarly, E17.5 mutant embryos had an increased number of stage 1-3 hair follicles than controls and 7.4% stage 4 follicles compared with none in the control (Fig. 2G,H; supplementary material Fig. S2E,F; Table 1).

Mutant hair follicles were larger than those of the control at E15.5-17.5 (Fig. 3G) and when compared with the stage 4 matched control follicles (Fig. 3H). The enlarged follicles expressed markers of hair follicle differentiation, such as keratin 17, and Sox9 for the outer root sheath (McGowan and Coulombe, 1998; Vidal et al., 2005), comparable to the control (Fig. 3I-L). Hair follicles in control ventral skin lacked precortex keratins recognized by the AE13 antibody in the stage 2 and 3 follicles (Fig. 3M) and AE13 immunoreactivity was first visible in stage 5 follicles at post-natal day (P) 0 (Paus et al., 1999) (data not shown). By contrast, at E17.5, we found clear AE13 immunoreactivity in the enlarged stage 4 mutant follicles (Fig. 3N), demonstrating accelerated maturation of hair follicles in the conditional β-catenin\textsuperscript{ex(3β)}/+ mutant skin.

Although the cellular organization in the mutant hair follicle seems abnormal, the nascent hair matrix cells interact comparably to those of controls as visualized by the response to Wnt signaling of Lef1 protein expression (supplementary material Fig. S2I,J). Similar to control skin, we found mature dermal papilla cells that were Sox2 positive at E15.5 (Fig. 3O,P) and alkaline phosphatase positive at E17.5 (supplementary material Fig. S2M,N) in the mutant skin (Driskell et al., 2009; Paus and Cottere, 1999). We also found that the large follicles were associated with dramatically enlarged dermal papilla (Fig. 3O,P; supplementary material Fig. S2M,N). Cell adhesion as judged by histological morphology (Enshell-Seijffers et al., 2010) of the dermal papilla cells appeared comparable in control and mutant embryos (supplementary material Fig. S2E,F). Dermal sheath formation detected by α-SMA staining was also comparable in mutant and control hair follicles (supplementary material Fig. S2K,L).

Taken together, these data demonstrate that sustained cell-autonomous β-catenin activity in the dermis during skin differentiation results in increased hair follicle size and number, accelerated hair follicle differentiation and enlarged dermal papilla.

**Sustained β-catenin activity in the dermis leads to enlarged placode and dermal condensate domains**

Next, we determined whether sustained activation of β-catenin in the developing dermis leads to expansion of hair placode and dermal condensate domain at the initiation stage. We analyzed known components of the Wnt, Bmp and Hh pathways involved in

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**Table 1. Numbers and stages of hair follicles from E13.5 to E17.5 in control HoxB6Cre-ER\textsuperscript{T1/+}; R26R/+ and mutant HoxB6Cre-ER\textsuperscript{T1/+}; R26R/+; β-catenin\textsuperscript{ex(3β)}/+ embryos**

<table>
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<th>Age/genotype</th>
<th>Stage 1</th>
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<th>Stage 4</th>
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<tr>
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<tr>
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<td>0</td>
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<td>64</td>
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<tr>
<td>E14.5 mutant</td>
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<td>0</td>
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<td>108</td>
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<tr>
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Hair follicles were counted from 30 fields at 4× magnification.
hair follicle initiation events (Millar, 2002; Schmidt-Ullrich and Paus, 2005) in both control and mutant embryos at E14.5. The secreted Wnt inhibitor Dkk1 was expressed in the dermis surrounding the hair follicle placodes and dermal condensate in control ventral skin at E14.5 (Fig. 4A) (Andl et al., 2002). Compared with control skin, we found expansion of the Dkk1 domain in the surrounding mesenchyme in response to sustained activation of β-catenin in the dermis of the mutants (Fig. 4B). Dkk4 mRNA expression in the preplacodes was also expanded in the ventral epidermis of the mutant embryos compared with controls (Fig. 4C,D). Bmp4 and Bmp7 mRNA expression was upregulated and expanded in the dermal condensate of mutant skin during the early stages of hair follicle development (Fig. 4E,F; supplementary material Fig. S2G,H) (Botchkarev et al., 1999; Wilson et al., 1999). Shh and Ptc1 showed expanded expression domains of both hair follicle placode and dermal condensate in the mutants compared with the littermate controls (Fig. 4G-J) (St-Jacques et al., 1998). Consistent with these results, Wnt signaling reporter activity showed an expanded domain in the enlarged epidermal placodes and dermal condensates (Fig. 4K,L). Thus, sustained dermal β-catenin transcriptional activity prior to hair follicle initiation leads to expansion of hair follicle and dermal condensate domains, although whether this effect is direct or indirect remains unknown.

Dermal Wnt/β-catenin activity after E12.5 regulates proliferation

The gain-of-function approach in the dermis yielded phenotypes that prompted us to develop an appropriate loss-of-function model to test the requirement of dermal β-catenin activity during fibroblast differentiation and hair follicle initiation. In our previous studies, deletion of β-catenin occurred in prespecified mesenchyme by E10.5 and led to the loss of the dermal fibroblast lineage (Atit et al., 2006; Ohtola et al., 2008). Here, we dissected the role of dermal canonical Wnt signaling activity in specified dermal fibroblasts after E12.5 by taking advantage of the highly efficient deletion of β-catenin by En1Cre (En1Cre/+; R26R/+; β-catenin<sup>fl/fl</sup>) (Brault et al., 2001; Kimmel et al., 2000; Soriano, 1999) in the dorsolateral dermal progenitors after E12.5 (Fig. 5A,B). The later deletion of β-catenin, after E12.5, did not alter the histology of the skin by E14.5 (Fig. 5C,D). Dermal density did not differ significantly between controls and mutants at E13.5 or E14.5 (Fig. 5Z; n = 3 embryos, six fields at 40× magnification per embryo). Compared with control skin, dermal fibroblasts in En1Cre/+; R26R/+; β-catenin<sup>fl/fl</sup> mutant appeared to be more loosely distributed at E16.5-P and the dermal layer was thinner at P0 (Fig. 5E-H).

At E13.5-14.5, in contrast to the control, there was an absence of Wnt signaling reporter activity, Twist2 or Fgrl1 expression in the mutant (Fig. 5I-P). There was no detectable difference in keratin 14 expression or in the gross morphology of the epidermis (Fig. 5G,H,Q,R), or in vasculature formation and innervation of the dorsolateral embryonic dermis (Fig. 5S-V). Compared with controls, mutant dermal fibroblasts were capable of expressing Col1a1 at E16.5, although the level of Col1a1 mRNA appeared to be reduced in the thinning dermis (Fig. 5W,X).

Consistent with the findings for mutant skin, deletion of dermal Wnt signaling/β-catenin activity led to a significant decrease in the proliferation index of dermal fibroblast progenitors in the upper dermis at E14.5 and E16.5. Compared with 46±4.3% at E14.5 and 26±7.0% at E16.5 proliferating upper dermal fibroblast cells in the control, the mutant had 34±3.88% and 13±6.9% in the equivalent region, respectively (Fig. 5Y). Upper cell density was comparable between control and mutant skin at E13.5 and E14.5, whereas it showed a significant decrease in the mutant at E16.5 (Fig. 5Z). However, no apoptosis of dermal fibroblasts was detectable by TUNEL assay in En1Cre/+; R26R/+; β-catenin<sup>fl/fl</sup> mutants and control littermates in this region between E14.5 and E16.5 (data not shown).

These data demonstrate that, after E12.5, dermal β-catenin activity functions to regulate upper dermal fibroblast proliferation of the embryonic dermis but not cell density during hair follicle initiation.

Dermal β-catenin activity is required for hair follicle initiation

Next, we tested the hypothesis that dermal β-catenin activity is required for hair follicle initiation. The earliest indicator of hair follicle initiation sites in the epidermis can be visualized by β-gal staining in two different Wnt signaling reporter mice: Conductin-lacZ and TCF/Lef-lacZ (Fig. 1B,C) (Zhang et al., 2009). In the absence of dermal β-catenin after E12.5 in the dorsolateral region of mutant embryos, Wnt signaling reporter activity was downregulated in the dorsolateral upper dermis at E13.5 and absent in both the upper dermis and the overlying ectoderm at E14.5 (Fig. 5N,P). Dkk4, Wnt10b and Edar are expressed in the preplacode in the ectoderm of the dorsal skin starting at E13.5-14.5 (Bazzi et al., 2007; Headon and Overbeek, 1999; Reddy et al., 2001) (Fig. 6A,C,E). In the absence of dermal β-catenin activity, Dkk4, Wnt10b and Edar mRNAs were absent from the surface ectoderm (Fig. 6B,D,F). Normally, Left1 is expressed initially throughout the epidermis, and then undergoes patterned upregulation early in the hair follicle placodes and dermal condensate (DasGupta and Fuchs, 1999; Kratochwil et al., 1996) (Fig. 6G). In the absence of dermal β-catenin, we found that Left1 expression was sustained broadly in the epidermis but was not patterned in the ectoderm and was absent in the dermis (Fig. 6H). In the controls, Shh also had patterned upregulation in the hair follicle placodes; Ptc1 and Bmp4 were expressed in the underlying dermal condensate (Fig. 6I,K,M). By contrast, Shh, Ptc1 and Bmp4 were absent in the mutant skin (Fig. 6J,L,N).

These data together demonstrate that dermal β-catenin activity is required for hair follicle placode initiation and dermal condensate formation. The absence of these earliest markers in the placode and dermal condensate indicate that these are downstream events from dermal β-catenin activity.

Epidermal Wnt ligands are required for canonical Wnt signaling activity in dermal fibroblast progenitors

We next tried to define the tissue source of dermal Wnt signaling activity. Our data showed that Wls was broadly expressed in the epidermis (Fig. 1D-G). To test whether epidermal Wnt ligands are required for Wnt signaling activity during dermal development, we conditionally deleted Wls in the surface epidermis with Keratin14-Cre (K14Cre) (Carpenter et al., 2010; Dassule et al., 2000; Soriano, 1999). The K14Cre/+; R26R/+; Wls<sup>fl/fl</sup> mutant embryos showed open eyelids and loss of nail formation at E18.5 compared with littermate controls (supplementary material Fig. S3A,B). K14Cre evenly recombinated the R26R allele and expressed β-gal in the dorsal surface epidermis by E13.5 (supplementary material Fig. S3C-E). Unlike control embryonic skin, Wls protein expression in the mutant embryos was barely detectable in the epidermis from E13.5 onwards (supplementary material Fig. S3F-K). Histological analysis of the skin from E14.5-18.5 showed loss of hair follicle
formation in the mutant embryos (Fig. 7A-C'). Axin2 was present in the upper dermal fibroblast of controls at E14.5 but absent in mutant skin (Fig. 7D,D'). Similarly, other Wnt signaling target genes such as Wnt11 (Christiansen et al., 1995), Twist2 and Fgfr1 mRNA was present in the upper dermis of control but absent in mutant skin. (M-P) Wnt reporter activity showed expression in the upper dermis and patterned upregulation in the placodes and dermal condensates in controls, whereas reporter activity was absent in the epidermis and dermis of mutant skin. (Q-X) K14, PGP9.5, Pecam1 and Col1a1 mRNA expression revealed comparable epidermal basal keratin expression, innervation, vasculature formation and collagen production, respectively, in control and mutant skin. Dashed lines demarcate the epidermal-dermal boundary. Control and mutant skin sections were photographed at the same magnification. (Y) The percentage of BrdU* proliferating dermal fibroblasts in the upper dermis was significantly decreased in the mutant compared with the control. (Z) Cell density in the upper dermis showed no difference between control and mutant at E13.5-14.5, but a significant decrease in the mutant embryos at E16.5. Error bars indicate s.d. dc, dermal condensate; hf, hair follicle.

Fig. 5. Dermal Wnt/β-catenin activity regulates dermal fibroblast proliferation. (A, B) X-Gal staining shows En1Cre lineage-marked cells in the dermis at E12.5 and E13.5. (C-F) X-Gal staining of control (C,E) and mutant (D,F) embryonic mouse dorsolateral skin. (G,H) Hematoxylin and Eosin (H&E) staining of perinatal skin revealed a loose distribution of dermal fibroblasts and a thinner dermal layer in the mutant. (I-L) Twist2 and Fgfr1 mRNA was present in the upper dermis of control but absent in mutant skin. (M-P) Wnt reporter activity showed expression in the upper dermis and patterned upregulation in the placodes and dermal condensates in controls, whereas reporter activity was absent in the epidermis and dermis of mutant skin. (Q-X) K14, PGP9.5, Pecam1 and Col1a1 mRNA expression revealed comparable epidermal basal keratin expression, innervation, vasculature formation and collagen production, respectively, in control and mutant skin. Dashed lines demarcate the epidermal-dermal boundary. Control and mutant skin sections were photographed at the same magnification. (Y) The percentage of BrdU* proliferating dermal fibroblasts in the upper dermis was significantly decreased in the mutant compared with the control. (Z) Cell density in the upper dermis showed no difference between control and mutant at E13.5-14.5, but a significant decrease in the mutant embryos at E16.5. Error bars indicate s.d. dc, dermal condensate; hf, hair follicle.
Dermal β-catenin activity for skin development

**Fig. 6. Patterned upregulated expression of hair follicle initiation markers was absent without dermal Wnt signaling/β-catenin activity.** (A-F) In situ hybridization for Dkk4, Wnt10b and Eda mRNA revealed patterned upregulated expression in the control (A,C,E) but an absence of expression in the mutant mouse skin (B,D,F). (G,H) Expression of Lef1 in the epidermis and focal upregulation in the hair follicle placode and dermal condensate in the control, whereas patterned expression of Lef1 was absent in the mutant skin. (I-N) Ptc1, Shh and Bmp4 mRNA expression was observed at sites of hair follicle initiation in control skin (I,K,M), but was absent in mutant skin (J,L,N). Dashed lines indicate the dermal-epidermal boundary. Downward arrows indicate placodes in the epidermis and the upward arrows indicate dermal condensates. All images are at the same magnification.

K14Cre; R26R; Wlsfl/fl mutants (P=9.0×10⁻⁵) (Fig. 7H). Thus, ectodermal Wnt ligand secretion is required to activate the canonical Wnt signaling response and regulates proliferation of the underlying upper dermal fibroblasts.

Given the expression of Wnt5a, Wnt10a and Wnt11 mRNAs in the upper dermis and dermal condensate at E14.5 (Reddy et al., 2001), we also tested whether Wls in the dermal fibroblast progenitors is required for Wnt signaling activity and responsiveness in the dermis. En1Cre/+; R26R/+; Wlsfl/fl mutant embryos were generated in which Wls was deleted in the dorsolateral dermal mesenchyme by the En1Cre line after E12.5. Compared with the controls, Wls expression was absent in the dermal condensate but maintained in the epidermis at E14.5 in the En1Cre/+; R26R/+; Wlsfl/fl mutant (Fig. 7K,K'). Moreover, our quantitative PCR results revealed a significant reduction of Wls expression in the dorsal dermis of En1Cre/+; R26R/+; Wlsfl/fl mutant embryos (Fig. 7O). The dermis was histologically indistinguishable between control and mutant embryos at E14.5 and E17.5 (Fig. 7J,J',N,N'). Expression of Twist2 mRNA, dermal Lef1 protein and markers of innervation (PGP9.5; Uch1 – Mouse Genome Informatics), vasculature formation (Pecam1), hair follicles (keratin 17) and epidermal differentiation (keratin 10, filaggrin) was comparable between heterozygote littermate controls and En1Cre/+; R26R/+; Wlsfl/fl mutant embryos at E14.5 (Fig. 7L-M; supplementary material Fig. S4M-V). These data together clearly demonstrate that epidermal Wnt ligands, and not mesenchymal Wnt ligands, are required for Wnt signaling responsiveness in the upper dermis and for subsequent hair follicle initiation.

**DISCUSSION**

Wnt signaling/β-catenin activity plays diverse roles in the development and patterning of embryonic skin. However, the in vivo function of dermal Wnt signaling/β-catenin activity during skin development remains largely uncharacterized. Here we have used various spatiotemporally restricted genetic tools to define the source and function of dermal β-catenin activity during skin development. We show that in response to epidermal Wnt ligands, dermal Wnt signaling/β-catenin activity regulates the proliferation of dermal fibroblast progenitors. Our data also clearly demonstrate that β-catenin activity in the upper dermis is obligatory for all the early events leading to epidermal patterning and hair follicle initiation. Our data support a reciprocal signaling loop model in which Wnt ligands from the epidermis result in uniform β-catenin activity in the upper dermis, which is required to generate the dermal signal crucial for the patterned expression of epidermal β-catenin and hair follicle initiation (Fig. 7P).

Wnt signaling reporter activity suggests that there might be three temporally and functionally distinct roles for dermal β-catenin activity. In the first window, Wnt signaling activity regulates the survival (E9.5-10.5) and specification (E10.5-11.5) of dermal fibroblast progenitors (Atit et al., 2006; Ohtola et al., 2008; Olivera-Martinez et al., 2001; Tran et al., 2010). In the second window, upper dense dermis formation in the chick (E6) and mouse (E12.5-13.5) skin requires proliferation and movement of dermal fibroblasts (Dhouailly et al., 2004; Sengel and Mauger, 1976). We show that markers of the dermal fibroblast lineage in the upper dense dermis, such as Twist2, Wnt11 and Fgf1 mRNA expression, are responsive to Wnt signaling activity in the mouse upper dermis. Stabilizing dermal β-catenin activity leads to expansion of the domain exhibiting a canonical Wnt response and to an increase in fibroblast proliferation and cell density in the upper dermis. The data presented here show that deletion of dermal β-catenin or epidermal Wls after E12.5 leads to an absence of Wnt responsiveness in the upper dermis and to a decrease in the fibroblast progenitor proliferation by E14.5, without altering the cell density and survival of the upper dermis during the hair follicle initiation stages. Therefore, in this second role, dermal Wnt responsiveness/β-catenin activity is required to regulate the proliferation but not the cell density of upper dermal fibroblasts. In the third window, during hair follicle initiation (E13.5-14.5), the molecular identity of the dermal contribution has remained elusive (Millar, 2002). Here, we show that the uniform dermal Wnt signaling activity at E12.5-13.5 is required to generate the as-yet-undefined first dermal signal.

We show that dermal β-catenin activity requires epidermal Wnt ligand secretion. The initially uniform β-catenin activity in dermal precursors starting at E11.5 is likely to be a response to the earlier uniform expression of epidermal Wnt ligands (Andl et al., 2002;
Fig. 7. Dermal canonical Wnt signaling activity depends on epidermal but not dermal Wnt ligands. (A-C') Transverse sections at the forelimb level from E14.5-18.5 control and mutant mouse embryos. (D-G') Axin2, Twist2, Fgfr1 mRNA expression and Lef1 protein expression revealed loss of Wnt responsiveness in the upper dermis of the mutant compared with the control. Note that Lef1 expression remained intact in the basal layer of the epidermis of the control and mutant skin (G,G'). (H) The percentage of BrdU* proliferating dermal fibroblasts was significantly reduced in the mutant compared with the control. (I) Cell density in upper dermis showed no difference between control and mutant at E13.5-14.5, but a significant decrease in the mutant embryos at E16.5 and E18.5. (J-N') Transverse sections of skin at the forelimb level from control (J-N) and mutant (J'-N') skin. Skin samples were stained with H&E (J,J'), for Wls protein (K,K'), Twist2 mRNA (L,L') or Lef1 protein (M,M'), or for β-gal activity (N,N'). Arrows indicate dermal fibroblasts with positive nuclear Lef1 immunoreactivity. White dashed lines demarcate the epidermal-dermal junction. Control and mutant skin sections are at the same magnification. (O) The relative level of Wls mRNA in the dorsal dermis detected by quantitative RT-PCR showed a significant reduction in the mutant embryo compared with control. There was no discernible difference in morphology, Wnt responsiveness or hair follicle formation between control and mutant skin. Error bars indicate s.d. (P) Proposed model for the reciprocal signaling loop whereby Wnt ligands are first secreted from the epidermis and lead to the activation of canonical Wnt signaling/β-catenin pathway in the underlying dermal fibroblasts, which then send signals back to the epidermis. The epidermis then becomes able to respond to epidermal Wnt ligands and to generate patterned upregulated β-catenin activity in the preplacode. Subsequently, β-catenin-expressing epidermal cells in the placode send signals to recruit dermal fibroblast cells to form the dermal condensate. DC, dermal condensate; hf/HF, hair follicle.
Chang et al., 2004; DasGupta and Fuchs, 1999; Reddy et al., 2001; Rodriguez-Niedenfuhr et al., 2003; Wagner et al., 2000). Wnt5a, Wnt10a and Wnt11 are expressed only in the upper dermis at E14.5, and deletion of Wls in the dermis eliminates functional redundancy between the Wnt ligands (Reddy et al., 2001). Elimination of dermal Wnt ligand secretion did not alter Wnt responsiveness or Colla1 mRNA expression in the upper dermis, innervation, vasculature formation, epidermis stratification or embryonic hair follicle development. Dermal Wnt5a-deficient mouse skin shows hair follicle differentiation defects starting 1 week after grafting on nude mice (Hu et al., 2010). However, mutant mice do not survive postnatally, which prevents further investigation of late stage hair follicle differentiation and renewal. Thus, dermal Wnt ligands appear to be dispensable for embryonic dermal fibroblast development and hair follicle initiation. Determining which of the broadly expressed Wnt ligands in the ectoderm are functionally required for uniform and patterned dermal β-catenin activity will further elucidate the components of the reciprocal signaling network that operates between the epidermis and dermal fibroblasts.

Previous studies have indicated that the dermal fibroblasts induce patterning of the hair follicle placode array by initiating expression of activating and inhibitory factors (Hardy, 1992; Millar, 2002; Schmidt-Ullrich and Paus, 2005). Our results demonstrate that uniform dermal β-catenin activity is required to generate the necessary signal from the dermis. These data are consistent with the requirement of mesenchymal Lef1, a component of the Wnt signaling pathway, in whisker follicle initiation (Kroatchwil et al., 1996). Deletion of epidermal β-catenin leads to loss of hair follicle placode formation and of all the other hair follicle initiation events (Huelsken et al., 2001; Zhang et al., 2009). Here, we show that the first patterned epidermal β-catenin activity is dependent on dermal β-catenin activity.

In the chick skin, it was hypothesized that dense dermis formation is required for generating sufficient signal to initiate placode formation in the epidermis (Sengel and Mauger, 1976). Based on a reconstitution assay, Jiang and colleagues proposed that formation of dermal microaggregates needs to achieve a cell density threshold before the induction of feather primordia through a reaction-diffusion mechanism (Jiang et al., 1999). However, our data show that stabilized β-catenin leads to a significant increase in upper dermis cell density at E13.5 without inducing premature formation of hair follicles, suggesting that the increased cell density is not sufficient for hair follicle initiation. Conditional deletion of either dermal β-catenin activity or epidermal Wnt ligand secretion after E12.5 does not immediately affect the cell density in the upper dermis at E13.5 or E14.5. Therefore, whether the formation of the upper dense dermis is required for hair follicle initiation remains unknown. Taken together, our data support that dermal Wnt/β-catenin signaling activity is required for hair follicle induction. However, whether cell density in the upper dermis is required for hair follicle induction is not known in our mouse model.

Stabilizing β-catenin leads to changes in the dermis and subsequently to the formation of larger placodes and dermal condensates in the skin. In the reconstitution assay, there was a trend of increased feather primordia size when the number of dermal fibroblasts increased, although the change was not statistically significant (Jiang et al., 1999). Therefore, in our stabilized dermal β-catenin mutants, the larger dermal condensates and hair follicles could either be caused by an increase in activating factors or due to an increase in the number of dermal fibroblasts. To elucidate the mechanism that drives the formation of larger hair follicle and dermal condensate in the mutant embryos, reconstitution assays will be required with similar cell density using E13.5 control and stabilized β-catenin dermal fibroblasts in the upper dermis.

The formation of dermal condensates follows placode initiation in the epidermis (Hardy, 1992). Therefore, the loss of dermal condensate in dermal β-catenin deletion mutants might occur due to the absence of hair follicle placodes. Forced activation of dermal β-catenin was not sufficient to convert all dermal fibroblasts to a dermal condensate fate. In contrast to our results, sustained embryonic epidermal β-catenin activity was sufficient to convert upper dermis to dermal condensate fate, suggesting that epidermal signals induce dermal condensate fate (Zhang et al., 2008). However, a recent study showed that sustained epidermal β-catenin activity could induce mature adult dermal fibroblasts to resemble a neonatal state with a high cell density (Collins et al., 2011), which implies different roles for epidermal β-catenin. Consistent with our proposed model from the loss-of-function studies, sustained dermal β-catenin activity might be required to generate a secondary signal that favors the dermal condensate fate in the developing dermal fibroblasts. In postnatal skin, β-catenin activity in dermal papilla is required to generate signals that promote the proliferation of adjacent matrix cells in the hair follicle (Enshell-Seijffers et al., 2010). We found that sustained dermal β-catenin activity in the larger dermal papillae and adjacent dermal fibroblast cells led to accelerated differentiation of precortex cells in the follicles of the mutant skin. The effects on epidermal differentiation are likely to be due to secreted factors activated by sustained cell-autonomous mutant β-catenin and amplified responses by the epidermis.

Deletion of epidermal β-catenin activity does not affect the uniform dermal Wnt signaling/β-catenin activity (Zhang et al., 2009) and here we show that dermal Wnt ligand secretion is dispensable for hair follicle initiation. These data suggest that epidermal Wnt ligand secretion is not dependent on epidermal or dermal Wnt signaling/β-catenin activity. Thus, it is most likely that patterned β-catenin activity in the epidermis occurs in response to the first wave of Wnt ligands or other factors in the ectoderm. Here we proposed that the following events occur during hair follicle initiation (Fig. 7P); (1) uniform epidermal Wnt ligands induce upper dermal Wnt/β-catenin activity and regulate fibroblast proliferation; (2) epidermal keratinocytes respond to a putative signal generated by this uniform upper dermal Wnt/β-catenin activity; (3) patterned Wnt signaling/β-catenin activity is upregulated by Wnt ligands secreted by epidermal keratinocytes; (4) hair follicle placodes are formed in response to the collective sum of activating and inhibitory effects at a given time and space. How the uniform dermal β-catenin activity initiates patterned upregulation of Wnt signaling/β-catenin activity in the epidermis remains elusive and will be the focus of future studies. Taken together, our results support the model that uniform dermal β-catenin activity is required for hair follicle initiation and is dependent on epidermal Wnt ligands (Fig. 7P).

Our results from studying the loss and gain of Wnt signaling activity in dermal fibroblasts have implications for human dermal diseases and tissue engineering of dermis that can induce hair follicles. Attenuated Wnt signaling is associated with congenital human focal dermal hypoplasia and activation of the pathway has been linked to skin fibrosis and fibroproliferative diseases (Bayle et al., 2008; Bowley et al., 2007; Grzeschik et al., 2007; Wang et al., 2007). Manipulating Wnt signaling activity in the adult dermis will provide insight into the etiology of dermal diseases. Defining the Wnt signaling signature in dermal fibroblasts will allow us to develop new therapeutic strategies for dermal diseases and hair follicle regeneration.
The authors declare no competing financial interests.

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