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Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles

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In hair follicle development, a placode-derived signal is believed to induce formation of the dermal condensation, an essential component of ectodermal organs. However, the identity of this signal is unknown. Furthermore, although induction and patterning of hair follicles are intimately linked, it is not known whether the mesenchymal condensation is necessary for inducing the initial epithelial pattern. Here, we show that fibroblast growth factor 20 (Fgf20) is expressed in hair placodes and is induced by and functions downstream from epithelial ectodysplasin (Eda)/Edar and Wnt/β-Catenin signaling to initiate formation of the underlying dermal condensation. Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles and subsequent formation of guard, awl, and auchene hairs. Although primary dermal condensations are absent in Fgf20 mutant mice, a regular array of hair placodes is formed, demonstrating that the epithelial patterning process is independent of known histological and molecular markers of underlying mesenchymal patterns during the initial stages of hair follicle development.

[Keywords: Eda signal; Fgf20; Wnt signal; dermal condensation initiation; hair follicle development; mesenchymal–epithelial interactions]

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hair follicles. These studies also lead to the unexpected conclusion that epithelial and mesenchymal morphogenesis can be uncoupled during the initial steps of hair follicle development.

Results

Canonical Eda/Edar and Wnt/β-Catenin signals regulate Fgf20 expression in vivo and in vitro

While screening for epithelial factors that are regulated by the Eda/Edar pathway, we identified Fgf20 as a gene rapidly induced following treatment of Eda<sup>−/−</sup> (Tabby mutant) skin explants with recombinant EDA (Lefebvre et al. 2012). The Fgf20 message was increased 3.3-fold and 16-fold following treatment with EDA for 2 and 4 h, respectively, compared with untreated controls (Fig. 1A). Analysis of Fgf20 expression using an Fgf20-β-galactosidase (βGal) knock-in allele [Fgf20<sup>βGal</sup>] (Huh et al. 2012) revealed focal Fgf20 expression as early as embryonic day E13.5 (E13.5), prior to the appearance of morphologically distinct primary hair placodes (Fig. 1B; Supplemental Fig. S1A). By E14.5, Fgf20<sup>βGal</sup> expression was prominent in the placodal epithelium in a pattern that matched Edar (Laurikkala et al. 2002) and Fgf20 expression, as determined by in situ hybridization and βGal staining (Fig. 1B; Supplemental Fig. S1B,G). Fgf20<sup>βGal−/−</sup> expression was detected throughout embryonic hair follicle morphogenesis in all pelage hair types (Supplemental Fig. S1A–F). To examine whether Fgf20 was regulated in vivo by Eda/Edar, we bred gain-of-function and loss-of-function alleles of Eda onto the Fgf20<sup>βGal−/−</sup> background and stained for βGal activity. Activation of Eda in epidermis [K14-Eda and K14-Edar] resulted in increased Fgf20<sup>βGal</sup> activity, while loss of Eda/Edar signaling [Eda<sup>−/−</sup>βGal<sup>−/−</sup>] resulted in decreased Fgf20<sup>βGal</sup> activity (Fig. 1B; Supplemental Fig. S2A,B).

Fgf20 was previously implicated as a downstream target of β-Catenin (Chamorro et al. 2005), and, as anticipated, transfection of a β-Catenin-LEF1 fusion protein increased the expression of a murine Fgf20 promoter–luciferase reporter by 65-fold (Fig. 1C). Consistent with these in vitro studies, in vivo activation [expression of the β-CAT<sub>4Exc</sub> allele] or inhibition [conditional inactivation of β-Catenin using K14-cre] of epithelial Wnt/β-Catenin signaling resulted in gain and loss of Fgf20<sup>βGal</sup> expression, respectively (Fig. 1D). These data indicate that epithelial Eda/Edar and Wnt/β-Catenin signals activate Fgf20 expression in vivo.

Fgf20 is required for guard hair formation

During embryogenesis, different pelage hair types are induced in successive waves. Guard (tylootrich) hair follicles are the first to form, followed by formation of awl, auchene, and zigzag follicles (Schmidt-Ullrich and Paus 2005). To determine whether Fgf20 is functionally important for hair follicle formation, we examined hair from back skin of adult Fgf20<sup>βGal−/−</sup> mice. Of the four morphologically distinct hair types, guard hairs were missing in Fgf20<sup>βGal−/−</sup> mice (Fig. 2A), while, awl, auchene, and zigzag hairs were readily identified and showed normal shaft morphology (Fig. 2B). To determine whether lack of Fgf20 affected the development of secondary and tertiary hair types, numbers of hairs from 3-wk-old Fgf20<sup>βGal−/−</sup> and Fgf20<sup>βGal+G−/−</sup> mice were quantified. In heterozygous Fgf20<sup>βGal+G−/−</sup> mice, guard hairs represented 2.2% ± 1.1% of the total population. In Fgf20<sup>βGal+G−/−</sup> mice, guard hairs were not detected (Fig. 2C). Interestingly, the percentage of awl and auchene hairs was significantly reduced from 9.2% ± 2.2% and 9.5% ± 3.1%, respectively, in Fgf20<sup>βGal+G−/−</sup> mice to 1.8% ± 1.9% and 2.8% ± 1.7%, respectively, in Fgf20<sup>βGal+G−/−</sup> mice [P = 0.002 and P < 0.009, n = 4, respectively] (Fig. 2C). The percentage of zigzag hairs was increased from 79% ± 3.5% in Fgf20<sup>βGal+G−/−</sup> mice to 95.4% ± 3.5% in Fgf20<sup>βGal+G−/−</sup> mice [P < 0.003] (Fig. 2C). Thus, Fgf20 is required for the formation of primary and most of the secondary hairs in mice.

Fgf20 is required for the primary dermal condensation

Prior to primary hair placode formation [E13.5], the skin of Fgf20<sup>βGal+G−/−</sup> embryos was histologically indistinguishable from Fgf20<sup>βGal+G−/−</sup> embryos (data not shown). At E14.5, scanning electron microscope analysis suggested the absence of primary hair follicle primordia in Fgf20<sup>βGal+G−/−</sup> embryos (Fig. 2D), yet epithelial thickenings (placodes)
were histologically evident in both Fgf20−/Gal−/+ and Fgf20−/Cal−/Gal−/+ embryos (Fig. 2E). Strikingly, there was no histological evidence of dermal condensation formation in Fgf20−/Cal−/Gal−/+ embryos (Fig. 2E). At E15.5, two types of hair follicles could be identified in Fgf20−/Cal−/Gal−/+ embryos—small and flat placodes (the majority) and follicles that had grown deeper into the dermis (occasional)—but none of them were associated with dermal condensations (Fig. 2E). At E16.5 in Fgf20−/Cal−/Gal−/+ embryos, the primary hair follicles reached the peg stage, and secondary hair placodes were formed. In contrast, in Fgf20−/Cal−/Gal−/+ embryos, most hair follicles were very small and were not associated with dermal condensations. However, sporadic primary hair placodes extended into the dermis to form a hair peg and were associated with a very small mesenchymal condensation (Fig. 2E). At E18.5 in Fgf20−/Cal−/Gal−/+ embryos, some primary hair follicles were observed and were occasionally bifurcated, indicating an additional defect in hair follicle development (Fig. 2E). Tertiary placodes formed in both genotypes (Fig. 2E). These data show that Fgf20 is necessary for the formation of dermal condensations in primary hair follicles.

To examine Fgf20 signaling in the dermis and look for molecular evidence of dermal condensation, phospho-

**Figure 2.** Loss of Fgf20 results in guard hair agenesis. (A) Image of 4-wk-old mice showing guard hair shafts sprouting from back skin in a Fgf20−/Cal−/+ mouse (arrow, left) but not in a Fgf20−/Cal−/Gal−/+ mouse (right). (B) Image of hairs from back skin of a Fgf20−/Cal−/+ (left) or Fgf20−/Cal−/Gal−/+ (right) mouse. (C) Quantification of hair types from 3-wk-old mice. Fgf20−/Cal−/+ mice show complete loss of guard hairs and decreased awl and auchene hairs. (D) Scanning electron micrograph showing primary hair follicle primordia as round protrusions in a Fgf20−/Cal−/+ embryo, but the surface of a Fgf20−/Cal−/Gal−/+ embryo appears flat. (E) Histology of E14.5, E15.5, E16.5, and E18.5 skin from Fgf20−/Cal−/+ and Fgf20−/Cal−/Gal−/+ embryos. Arrows indicate dermal condensations. (Bottom right panel) Note the bifurcated hair follicle. (a) Auchene; (g) guard; (awl) awl; (z) zigzag. Bar, 100 μm.
Dermal condensation induced by Fgf20

To assess the consequences of loss of Fgf20 and the absence of dermal condensation formation on hair placode development, we analyzed the expression of several key epithelial genes in Fgf20 Gal+/Gal− embryos at E14.0–E14.5 (Schmidt-Ullrich et al. 2006; Pummila et al. 2007). Unexpectedly, Wnt10b, a well-characterized hair placode marker, as well as Lef1 and β-Catenin showed a stripe-like expression pattern in the epidermis in Fgf20 Gal+/Gal− embryos [Fig. 4A–C]. Also, Fgf20 Gal− activity showed a similar pattern in Fgf20 Gal−/Gal− embryos, in contrast to the punctate expression seen in Fgf20 Gal+/Gal− embryos [Fig. 4D]. During hair follicle induction, Wnt/β-Catenin signaling is active in both the epidermis and dermis (DasGupta and Fuchs 1999; Zhang et al. 2008, 2009). In control embryos, Axin2, a target of canonical Wnt signaling, was expressed in the epidermis in primary hair follicles and in the underlying dermis. However, in Fgf20 Gal+/Gal− embryos, dermal expression of Axin2 was severely decreased, while epidermal expression was slightly broader and more intense [Fig. 4E]. Immunohistochemical detection of β-Catenin showed nuclear localization [Fig. 4F, arrows] in the dermal condensations of control embryos but reduced nuclear localization in Fgf20 Gal+/Gal− embryos [Fig. 4F]. Furthermore, nuclear Lef1 protein was reduced in the dermis of Fgf20 Gal+/Gal− embryos compared with littermate controls (Fig. 4G). These data indicate that Fgf20 inhibits canonical Wnt/β-Catenin signaling in the epidermis but activates it in dermal condensations.

Patterning of hair follicles is thought to be the result of a reaction–diffusion mechanism involving the interaction of diffusible substances that are commonly referred to as “morphogens.” In dermal condensation, the absence of primary dermal condensations in Fgf20 Gal+/Gal− embryos is expected because Fgf20 modulates Eda/Edar and Wnt/β-Catenin but not the Sonic hedgehog (Shh) signaling cascade.

Figure 4. Fgf20 modulates epithelial and mesenchymal Wnt/β-Catenin signaling during primary hair placode formation. (A–C) In situ hybridization for Wnt10b [A], Lef1 [B], and β-Catenin [C] showing stripe-like expression in Fgf20 Gal+/Gal− embryos compared with Fgf20 Gal−/Gal− embryos. (D) Fgf20 Gal− staining showing increased interfollicular βGal staining in Fgf20 Gal−/Gal− embryos compared with Fgf20 Gal−/Gal− embryos. (E) In situ hybridization of Axin2 showing increased expression in placodal cells but decreased expression in dermal cells in Fgf20 Gal+/Gal− embryos compared with Fgf20 Gal−/Gal− embryos. (F) Immunostaining for β-Catenin showing loss of β-Catenin nuclear localization (arrows) in dermal cells in Fgf20 Gal−/Gal− embryos compared with Fgf20 Gal−/Gal− embryos. (G) Immunostaining for Lef1 shows strong nuclear expression in the dermal condensation of Fgf20 Gal−/Gal− but not in Fgf20 Gal−/Gal− embryos at E14.5. (H, I) In situ hybridization for Dkk4 [H] and Sostdc1 [I] showing severely reduced expression in Fgf20 Gal−/Gal− embryos compared with Fgf20 Gal−/Gal− embryos. Bar, 100 μm.
to as “the activator” and “the inhibitor” [Kondo and Miura 2010]. Activating Wnt ligands and their soluble inhibitors (Dkk1 and Dkk4) have been proposed to function at the core of this patterning process [Andl et al. 2002; Sick et al. 2006]. The “spreading” of Wnt10b expression in Fgf20βGal/Bgal embryos is consistent with predictions of reaction–diffusion models in situations where activator concentrations become saturating [Mou et al. 2006; Kondo and Miura 2010]. Absence of Dkk1 [Fig. 3G] together with the severe down-regulation of Dkk4 [Fig. 4H] and Sostdc1 (Ectodin and Wise) [Fig. 4I], another Wnt antagonist regulating hair placode size [Närhi et al. 2012] in Fgf20βGal/Bgal embryos, may explain the observed phenomenon.

We next analyzed expression of Edar and observed severely decreased levels in Fgf20βGal/Bgal embryos compared with Fgf20βGal/+ embryos [Fig. 5A]. Ik-Bα, the downstream target of Eda/Edar (Schmidt-Ullrich et al. 2007; Pummila et al. 2007), was similarly decreased in Fgf20βGal/Bgal embryos [Fig. 5B]. Thus, loss of Fgf20 suppresses Edar expression, leading to reduced Edar signaling. Reduced expression of Edar was surprising given that placodal Wnts have been shown to up-regulate Edar expression [Zhang et al. 2009]. These data suggest that in addition to regulation by Wnts, Edar expression also depends on signals emanating from the dermal condensation.

Fgf20 functions downstream from Eda/Edar and Wnt/β-Catenin signaling to form dermal condensations

If Fgf20 is the critical signal for dermal condensation formation downstream from Wnt/β-Catenin and Eda/Edar signaling, then loss of Fgf20 would block formation of primary dermal condensations in Eda or Wnt/β-Catenin gain-of-function (K14-Eda or K14-Cre:β-CateninEx3) embryos. We addressed the signaling hierarchy between Fgf20 and Eda by comparing Fgf20βGal/+ and Fgf20βGal/C0 embryos with K14-Eda,Fgf20βGal/+ and K14-Eda,Fgf20βGal/C0 embryos. Overexpression of Eda resulted in enlarged hair placodes associated with larger Sox2+ dermal condensations (Fig. 6A; Supplemental Fig. S6), consistent with previously reported data [Mistone et al. 2004]. Interestingly, the size of the primary hair placodes in K14-Eda embryos (indicated by P-Cadherin staining) was unaffected by loss of Fgf20, yet dermal condensations were absent [Fig. 6A; data not shown]. The inability of excess Eda to rescue dermal condensation formation in K14-Eda; Fgf20βGal/C0 embryos could be due to reduced Edar expression due to the absence of Fgf20 [Fig. 5A]. However, we found robust induction of Ik-Bα expression by K14-Eda in both heterozygous and Fgf20βGal/Bgal embryos [Fig. 6B], excluding this possibility. These data support a model in which Fgf20 is required for dermal condensation formation downstream from Eda/Edar signaling.
Hair follicle formation has been the lack of knowledge on the details and exact order of the inductive events during growth of placodal cells. A major obstacle in deciphering a second dermal signal drives proliferation and down-chyme to induce formation of a dermal condensation, and signal is believed to pass from the placode to mesenchyme (Hardy 1992). An initial mesenchymal signal instructs ordered series of epithelial–mesenchymal interactions. Hair follicle induction is thought to proceed via an initial mesenchymal signal instructing ordered series of epithelial–mesenchymal interactions.

**Discussion**

Hair follicle induction is thought to proceed via an ordered series of epithelial–mesenchymal interactions (Hardy 1992). An initial mesenchymal signal instructs the epithelium to make a placode, a second, epithelial signal is believed to pass from the placode to mesenchyme to induce formation of a dermal condensation, and a second dermal signal drives proliferation and down-growth of placodal cells. A major obstacle in deciphering the details and exact order of the inductive events during hair follicle formation has been the lack of knowledge on the identity of these key signals. Histological studies on nascent whisker primordia indicated the presence of dermal condensations prior to epithelial thickenings (Van Exan and Hardy 1980). In contrast, some placode markers show a labile prepattern in Eda−/− embryos where no primary dermal condensations have been recognized (Laurikkala et al. 2002; Mou et al. 2006; Fliniaux et al. 2008; Zhang et al. 2009), suggesting an “epithelium-first” patterning process. On the other hand, a recent study showed that Wnt/β-Catenin activity (Axin2−/−) becomes patterned in both the epithelium and dermis simultaneously before the appearance of morphologically distinct placodes or condensations (Zhang et al. 2009). Thus, it is controversial as to whether patterning first arises in the epithelium or the mesenchyme.

Here, we show that in the absence of Fgf20, a regular array of primary hair placodes forms in the absence of discernible dermal condensations. Although we cannot rule out the existence of an unknown asymmetric signal in Fgf20 mutant dermis, based on the morphological evidence and the absence of a wealth of dermal condensation markers (Sox2, p21, Dkk1, Inhba, β-Catenin, Axin2, Bmp4, Corin, CD133), we find the presence of such a cue unlikely. Rather, we interpret our data to indicate that the embryonic epithelium—once committed to form hair—has the ability to generate periodicity and that patterning originates initially in the epithelium and can arise independently of dermal condensations. We propose that the primary inductive cue is uniform rather than patterned, a conclusion supported by functional experiments showing that periodic dermal Wnt/β-Catenin activity depends on epithelial β-Catenin (Zhang et al. 2009). Our conclusions appear seemingly contradictory to studies using...
mesenchymal–epithelial tissue chimeras, which indicate that hair patterning is under the control of the dermis [Kollar 1970; Dhouauly 1973]. However, these tissue recombination experiments can be explained by a mesenchymal-derived inductive cue, even if uniformly expressed, influencing the spacing of hair placodes; for example, by altering reaction–diffusion dynamics.

Until now, molecular mechanisms governing dermal condensation formation have been poorly defined [Sennett and Rendl 2012]. Previous studies have implicated Shh and platelet-derived growth factor in dermal morphogenesis, but in mice lacking either factor, dermal papillae form but are reduced in size [St-Jacques et al. 1998; Chiang et al. 1999; Karlsson et al. 1999]. This indicates a role in dermal papilla maturation/maintenance, a function that is thought to require the Bmp inhibitor Noggin [Woo et al. 2012]. Additionally, Wnts have been proposed to participate in the generation of dermal condensations [Zhang et al. 2009; Sennett and Rendl 2012]. However, forced activation of dermal β-Catenin is not sufficient to induce dermal cells to adopt a dermal condensation fate [Chen et al. 2012], while activation of epithelial β-Catenin is necessary for the formation of dermal condensations [Zhang et al. 2009; this study], implying involvement of a Wnt-dependent epithelial signal for the formation of dermal condensations. Here, we identify Fgf20, downstream from Eda/NF-κB and Wnt/β-Catenin, as an essential signal required for dermal condensation formation in primary and most secondary hair follicles. The absence of an effect on tertiary hair follicles suggests either redundancy with another Fgf ligand or that another, as yet unidentified, signal is required for tertiary hair follicle formation. The Fgf receptor mediating the Fgf20 signal is also not defined; however, Fgfr1 is a good candidate, as it is expressed at high levels in the upper dermis at the time of hair follicle induction [Chen et al. 2012]. Additionally, Fgfr2 is expressed at low levels in the developing dermis [Richardson et al. 2009] and therefore could have redundant function with Fgfr1. Further support for a key evolutionarily conserved role for Fgf20 signaling in epidermal appendage formation comes from the scaleless mutant chicken, which lacks almost all feathers and scales due to a nonsense mutation in Fgf20 [Wells et al. 2012]. In addition, in vitro studies have implicated Fgfs in dermal condensation formation in chicken dermis [Song et al. 2004; Lin et al. 2009].

It is well established that dermal condensation/papilla cells can induce hair follicle development even when combined with non-hair-forming epithelium, and thus the underlying mechanism is a focus of regenerative medicine research [Hardy 1992; Yang and Cotsarelis 2010]. However, therapeutic efforts to use dermal papilla cells have been hampered by their low abundance, difficulty in isolation, and loss of hair follicle-inducing properties in culture [Yang and Cotsarelis 2010]. These studies, which identify Fgf20 as a signal essential for dermal condensation formation, combined with the knowledge of how dermal cells acquire hair follicle-inducing capacity, will be useful in future attempts to treat hair loss.

Materials and methods

Mice

Fgf20βGal+, Eda+/−, K14-Eda, K14-Edar, β-CatF/F, β-CatEx3, K14-cre, and Sox2-GFP mice were crossed to generate Fgf20βGal/βGal, Eda+/−, K14-Eda;Fgf20βGal/βGal, K14-Edar;Fgf20βGal/βGal, K14-cre;β-CatF/F, Fgf20βGal/βGal;β-CatCKO, Fgf20βGal/βGal;K14-cre;β-CatEx3, Fgf20βGal/βGal;D-CatF/F, K14-cre;β-CatEx3, Fgf20βGal/βGal;D-CatCKO, and K14-Eda;Sox2-GFP mouse lines [Harada et al. 1999, Dassule et al. 2000, Braun et al. 2001, Laurikka et al. 2002, D’Amour and Gage 2003, Mustonen et al. 2004, Huh et al. 2012]. Mice were maintained on a mixed genetic background, and littermates were used as controls as indicated. All animal studies were carried out in accordance with the guidelines and approval from the Washington University Animal Studies Committee and Finnish National Board of Animal Experimentation.

Electron microscopy

Embryos were fixed in 4% paraformaldehyde in PBS over two nights; dehydrated in a graded series of 50%, 70%, 94%, and 100% ethanol for 30 min each, and subjected to critical point drying (Bal-Tech, CPD 030) and platinum coating (Quorum, Q150TS coater). Samples were viewed with FEI Quanta 250 scanning electron microscope.

Luciferase assay

The mouse Fgf20 promoter construct was generated by subcloning a 5.2-kb fragment of the proximal promoter region containing putative β-Catenin/TCF-binding sites into the firefly luciferase reporter plasmid pGL3 Basic [Promega]. For transfection, HEK293T cells were seeded at 3 × 105 cells per well in gelatin-coated six-well plates. The following day, 950 ng of luciferase reporter plasmid was cotransfected with 500 ng of an expression vector coding for dominant active Lef1–β-Catenin fusion protein [Wong et al. 2002] or corresponding empty vectors and 50 ng of Renilla luciferase vector pRL-TK [Promega] using Fugene 6 transfection reagent [Roche]. After 24 h, luciferase activities were measured as described [Finiaux et al. 2008]. Three independent experiments with triplicate samples were performed.

Quantitative RT–PCR (qRT–PCR)

E14.5 Eda−/− back skin were dissected, cut into halves along the midline, and maintained for 2 h [n = 12] or 4 h [n = 6] in a 30-μL hanging drop of DMEM, 10% FCS, glutamine, and penicillin–streptomycin. For each treatment, one half was used as a control, while the other half was supplemented with 250 ng/mL recombinant Fc-Eda-A1 [Gaide and Schneider 2003]. RNA isolation and qRT–PCR were done as previously described [Finiaux et al. 2008]. Transcript number was quantified by comparing the sample data against a dilution series of PCR products of the gene of interest. Expression of Fgf20 was normalized against Ranbp1.

βGal staining

Embryos at various stages of development were fixed overnight in Murasky’s fixative [National Diagnostics], washed three times in PBS, and incubated in βGal-staining solution [2 mM MgCl2, 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1 mg/mg X-Gal in PBS] until optimal color development was observed. Samples were washed in PBS, fixed in 10% formalin, and imaged under a dissecting microscope. For staining histo-
logical sections, samples were cryosectioned, washed with PBS, and incubated in βGal-staining solution. Embryos were photographed on an Olympus SZX12 stereo microscope.

Histology
Embryos or dissected tissues were fixed in 4% PFA overnight at 4 °C, dehydrated, embedded in paraffin, and serially sectioned. For plastic histology (E14.5), tissues were embedded in Historesin as specified by the manufacturer (Leica) and stained with haematoxylin and eosin.

Immunohistochemistry
Embryos were embedded in OCT, frozen, and cryosectioned at 12 μm. Alternatively, paraffin-embedded tissues were serially sectioned at 7 μm, dewaxed, rehydrated, and microwaved (650 W) for 10 min in 10 mM sodium citrate buffer [pH 6.0]. Sections were washed with PBS and blocked with 0.1% Triton X-100 and 0.5% donkey serum. Primary antibodies were added to the section and incubated in a humidified chamber overnight at 4 °C. Sections were washed and incubated with secondary antibody for 1 h at room temperature. Samples were washed three times with PBS, placed on cover slips with Vectashield (Vector Laboratories), and photographed using a Zeiss LSM 700 confocal microscope or Zeiss Axio Imager M2. The primary antibodies used were as follows: βGal [1:500, abcam], Sox2 [1:500 [Millipore] or 1:200 [Santa Cruz Biotechnology]], P-Cadherin [1:100 [gift from Dr. Takeichi]], and β-Catenin (1:200; Cell Signaling Technology), phospho-Erk1/2 (1:200; Cell Signaling Technology), phoshro-Erk1/2 (1:200, Cell Signaling Technology), and CD133 (1:500, Millipore).

In situ hybridization
For whole-mount in situ hybridization, embryos were fixed in 4% PFA overnight at 4 °C, washed in PBS, and dehydrated in increasing concentrations of methanol. Embryos were rehydrated, washed with hybridization solution, and incubated overnight with digoxigenin-labeled RNA probes. After washing, embryos were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase [Roche], and the color reaction was performed using alkaline phosphate substrate [Roche]. Alternatively, whole-mount in situ hybridization was performed with InSituPro robot (Intavis AG) as previously described [Fliniaux et al. 2008]. Embryos were photographed on an Olympus SZX12 stereo microscope. The probes used were as follows: Bmp4, p21, Dkk1, Inhba, Bmp7, Edar, Itf8a, Wnt10b, Fgf20, Axin2, Shh, Patched, Lef1, β-Catenin, Dkk4, and Sostdc1 (Ectodin) [Andl et al. 2002; Laurikkala et al. 2002; Mustonen et al. 2004; Schmidt-Ullrich et al. 2006; Fliniaux et al. 2008; Närhi et al. 2008]. Radioactive in situ hybridization was performed on paraffin sections according to standard protocols using probes labeled with 35S-UTP. Dark-field images were inverted, linearly thresholded, and combined with bright-field images in Adobe Photoshop CS4.

Hair and dermal condensation counting
Different types of hairs were counted from 3-wk-old mice. More than 200 hairs were counted from each genotype. Dermal condensations were counted using E14.5 whole-mount Bmp4 in situ staining samples.

Statistics
Nonparametric Mann-Whitney U-test was used for statistical analysis of luciferase assay data. qRT–PCR data were analyzed with the nonparametric Wilcoxon signed-rank test for paired samples. P < 0.05 was considered to be significant.

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