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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−/− (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 (Fgf20bGal) (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, Fgf20bGal expression was prominent in the placodal epithelium in a pattern that matched Edar (Laurikka et al. 2002) and Fgf20 expression, as determined by in situ hybridization and βGal staining (Fig. 1B; Supplemental Fig. S1B,G). Fgf20bGal 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 Fgf20bGal background and stained for βGal activity. Activation of Eda in epidermis (K14-Eda and K14-Edar) resulted in increased Fgf20bGal activity, while loss of Eda/Edar signaling (Eda−/−) resulted in decreased Fgf20bGal 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-CatA allele) or inhibition (conditional inactivation of β-Catenin using K14-cre) of epithelial Wnt/β-Catenin signaling resulted in gain and loss of Fgf20bGal 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 (tylotrich) hair follicles are the first to form, followed by formation of awl, aucthe, 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 Fgf20bGal/bGal mice. Of the four morphologically distinct hair types, guard hairs were missing in Fgf20bGal/bGal mice (Fig. 2A), while, awl, aucthe, 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 Fgf20bGal/+ and Fgf20bGal/bGal mice were quantified. In heterozygous Fgf20bGal/+ mice, guard hairs represented 2.2% ± 1.1% of the total population. In Fgf20bGal/bGal mice, guard hairs were not detected (Fig. 2C). Interestingly, the percentage of awl and aucthe hairs was significantly reduced from 9.3% ± 2.2% and 9.5% ± 3.1%, respectively, in Fgf20bGal/+ mice to 1.8% ± 1.9% and 2.8% ± 1.7%, respectively, in Fgf20bGal/bGal 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 Fgf20bGal/+ mice to 95.4% ± 3.5% in Fgf20bGal/bGal 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 Fgf20bGal/bGal embryos was histologically indistinguishable from Fgf20bGal/+ embryos (data not shown). At E14.5, scanning electron microscope analysis suggested the absence of primary hair follicle primordia in Fgf20bGal/bGal embryos (Fig. 2D), yet epithelial thickenings (placodes
were histologically evident in both Fgf20<sup>βGal+</sup> and Fgf20<sup>βGal/bGal</sup> embryos (Fig. 2E). Strikingly, there was no histological evidence of dermal condensation formation in Fgf20<sup>βGal/bGal</sup> embryos. At E15.5, two types of hair follicles could be identified in Fgf20<sup>βGal/bGal</sup> 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<sup>βGal+</sup> embryos, the primary hair follicles reached the peg stage, and secondary hair placodes were formed. In contrast, in Fgf20<sup>βGal/bGal</sup> 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<sup>βGal/bGal</sup> 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-Erk1/2, Sox2, Bmp4, p21, Inhba (InhibinA, previously known as activinβ), and Dkk1 expression was examined (Andl et al. 2002, Laurikkala et al. 2002, Schmidt-Ullrich and Paus 2005). At E14.5, phospho-Erk1/2 was detected in dermal cells as well as the epidermis of Fgf20<sup>βGal+/+</sup> embryos (Fig. 3A, left). In the absence of Fgf20, the phospho-Erk1/2 was not detected (Fig. 3A, right). Sox2 is one of the earliest genes expressed in dermal condensations of primary and secondary, but not tertiary (zigzag), follicles (Driskell et al. 2009). By E13.75, P-Cadherin and Wnt10b-positive epidermal foci could be detected in both Fgf20<sup>βGal+/+</sup> and Fgf20<sup>βGal/bGal</sup> embryos (Supplemental Fig. S3A), and, occasionally, P-Cadherin<sup>+</sup> foci were associated with Sox2<sup>+</sup> cells in Fgf20<sup>βGal/bGal</sup> embryos but not in Fgf20<sup>βGal+/+</sup> embryos (Supplemental Fig. S3A). At E14.0 and E14.5, Sox2 expression was readily observed in dermal condensations in Fgf20<sup>βGal+/+</sup> embryos. However, no Sox2 expression was detected in Fgf20<sup>βGal/bGal</sup> littermates at these stages (Fig. 3B,C). At E15.5, hair follicles were still devoid of Sox2 expression, in sharp contrast to control embryos (Fig. 3D). Consistent with the morphological absence of dermal condensations, focal expression of Bmp4, p21, Dkk1, and Inhba was also not detected in Fgf20<sup>βGal/bGal</sup> dermis at E13.75–E14.5. (Fig. 3F–I). Supplemental Fig. S3A shows the expression of Sox2, Bmp4, p21, Inhba, P-Cadherin, and Dkk1 in Fgf20<sup>βGal+/+</sup> and Fgf20<sup>βGal/bGal</sup> embryos (Fig. 3F–I).
Dermal condensation induced by Fg20

Fgf20 modulates Eda/Edar and Wnt/β-Catenin but not the Sonic hedgehog (Shh) signaling cascade

To assess the consequences of loss of Fg20 and the absence of dermal condensation formation on hair placode development, we analyzed the expression of several key epithelial genes in Fg20^BGal/BGal^ 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 Left1 and β-Catenin showed a stripe-like expression pattern in the epidermis in Fg20^BGal/BGal^ embryos (Fig. 4A–C). Also, Fg20^BGal^ activity showed a similar pattern in Fg20^BGal/BGal^ embryos, in contrast to the punctuate expression seen in Fg20^BGal/+^ 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 Fg20^BGal/BGal^ 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 Fg20^BGal/BGal^ embryos (Fig. 4F). Furthermore, nuclear Lef1 protein was reduced in the dermis of Fg20^BGal/BGal^ embryos compared with littermate controls (Fig. 4G). These data indicate that Fg20 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 fields.

### Figure 4.
Fgf20 modulates epithelial and mesenchymal Wnt/β-Catenin signaling during primary hair placode formation. (A–C) In situ hybridization for Wnt10b (A), Left1 (B), and β-Catenin (C) showing stripe-like expression in Fg20^BGal/BGal^ embryos compared with Fg20^BGal/+^ embryos. (D) Fgf20^BGal^ staining showing increased interfollicular βGal staining in Fg20^BGal/BGal^ embryos compared with Fg20^BGal/+^ embryos. (E) In situ hybridization of Axin2 showing increased expression in placodal cells but decreased expression in dermal cells in Fg20^BGal/BGal^ embryos compared with Fg20^BGal/+^ embryos. (F) Immunostaining for β-Catenin showing loss of β-Catenin nuclear localization (arrows) in dermal cells in Fg20^BGal/BGal^ embryos compared with Fg20^BGal/+^ embryos. (G) Immunostaining for Lef1 shows strong nuclear expression in the dermal condensation of Fg20^BGal/+^ but not in Fg20^BGal/BGal^ embryos at E14.5. (H, I) In situ hybridization for Dkk4 (H) and Sostdc1 (I) showing severely reduced expression in Fg20^BGal/BGal^ embryos compared with Fg20^BGal/+^ embryos. Bar, 100μm.
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 Fgf20<sup>Gal/Gal</sup> 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 Fgf20<sup>Gal/Gal</sup> embryos, may explain the observed phenomenon.

We next analyzed expression of Edar and observed severely decreased levels in Fgf20<sup>Gal/Gal</sup> embryos compared with Fgf20<sup>Gal/+</sup> embryos [Fig. 5A]. Iκ-Bα, the downstream target of Eda/Edar [Schmidt-Ullrich et al. 2006; Pummillia et al. 2007], was similarly decreased in Fgf20<sup>Gal/Gal</sup> 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.

Shh is expressed in hair placodes and functions to induce proliferation of hair placode epithelial cells, enabling growth into the dermis and the formation of the hair peg [St-Jacques et al. 1998; Chiang et al. 1999]. Expression levels and the pattern of Shh and its target gene, Patched1, were comparable in control and Fgf20<sup>Gal/Gal</sup> embryos at E14.5 [Fig. 5C,D, Supplemental Fig. S5A,B]. These data show that Shh signaling functions independently of Fgf20 and that, despite intact Shh signaling, down-growth of primary hair follicles was impaired in Fgf20<sup>Gal/Gal</sup> embryos.

We also noted that in Fgf20<sup>Gal/Gal</sup> embryos, Shh expression was confined to placodal cells, unlike Wnt10b and βGal [Fgf20<sup>Gal</sup>], whose expression expanded into the interfollicular epidermis [cf. Figs.4A,D and 5C]. Expression of Shh (and primary placode formation) is thought to require both Wnt/β-Catenin [Andl et al. 2002; Fuchs 2007] and Eda/Edar [Schmidt-Ullrich et al. 2006; Pummillia et al. 2007]. In Fgf20<sup>Gal/Gal</sup> embryos, Eda/Edar signaling is greatly reduced [Fig. 5A,B]. To address whether Shh expression and placode formation in Fgf20<sup>Gal/Gal</sup> embryos requires Eda, we generated mice deficient in both Fgf20 and Eda. Eda<sup>−/−</sup>Fgf20<sup>Gal/+</sup> and Eda<sup>−/−</sup>Fgf20<sup>Gal/Gal</sup> embryos lacked all expression of Shh at E14.5 [Supplemental Fig. 5C,D]. These data indicate that Eda signaling, although strongly reduced, is nevertheless necessary for Shh expression in Fgf20<sup>Gal/Gal</sup> embryos. We propose that the relatively normal patterning of Shh expression and morphological placodes in Fgf20<sup>Gal</sup> mutants is achieved via simultaneous activation of Edar and Wnt/β-Catenin pathways.

**Figure 5.** Fgf20 modulates Eda/Edar but not Shh signaling during primary hair placode formation. (A–B) In situ hybridization for Edar [A] and Iκ-Bα [B] showing decreased expression in Fgf20<sup>Gal/Gal</sup> embryos compared with Fgf20<sup>Gal/+</sup> embryos. (C,D) In situ hybridization of Shh [C] and Patched1 [D] showing unaltered expression in Fgf20<sup>Gal/Gal</sup> embryos compared with Fgf20<sup>Gal/+</sup> embryos. Bar, 100 μm.

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-Catenin<sup>fl/+</sup>]<sup>LacZ</sup> embryos. We addressed the signaling hierarchy between Fgf20 and Eda by comparing Fgf20<sup>Gal/+</sup> and Fgf20<sup>Gal/Gal</sup> embryos with K14-Eda,Fgf20<sup>Gal/+</sup> and K14-Eda,Fgf20<sup>Gal/Gal</sup> embryos. Overexpression of Eda resulted in enlarged hair placodes associated with larger Sox2<sup>+</sup> dermal condensations [Fig. 6A; Supplemental Fig. S6], consistent with previously reported data [Mustonen 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,Fgf20<sup>Gal/Gal</sup> embryos could be due to reduced Edar expression due to the absence of Fgf20 [Fig. 5A]. However, we found robust induction of Iκ-Bα expression by K14-Eda in both heterozygous and Fgf20<sup>Gal/Gal</sup> 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-signal is believed to pass from the placode to mesenchyme to make a placode; a second, epithelial (Hardy 1992). An initial mesenchymal signal instructs ordered series of epithelial–mesenchymal interactions.

Hair follicle induction is thought to proceed via an ordered series of epithelial–mesenchymal interactions [Hardy 1992]. An initial mesenchymal signal instructs ordered series of epithelial–mesenchymal interactions. Hair follicle induction is thought to proceed via an 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[²⁸Gal]), 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[²⁸Gal], 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 independent 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

**Figure 6.** Eda/Edar and Wnt/β-Catenin signaling-induced dermal condensation is Fgf20-dependent. (A) Coimmunostaining for Sox2 and P-Cadherin showing that Sox2⁺ dermal condensation induced by Eda overexpression [K14-Eda] has been abolished in Fgf20[²⁸Gal]/[²⁸Gal] embryos compared with Fgf20[²⁸Gal]/⁺ embryos at E14.5. (B) In situ hybridization for Iκ-Bα showing increased expression in K14-Eda and K14-Eda;Fgf20[²⁸Gal]/⁺ embryos at E14.5. (C) Coimmunostaining for Sox2 and P-Cadherin at E13.5 showing that Sox2⁺ dermal condensations induced by K14-Cre;β-Cat⁻¹[³⁸Ex3][²⁸Catact] were absent in Fgf20[²⁸Gal]/⁺ embryos compared with Fgf20[²⁸Gal]/⁺ embryos, yet premature induction of placodes was unaffected by loss of Fgf20 (cf. Supplemental Fig. S1A). Bar, 100 μm.
mesenchymal–epithelial tissue chimeras, which indicate that hair patterning is under the control of the dermis [Kollar 1970; Dhouailly 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 Rendell 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 Rendell 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, as a signal essential for dermal condensation formation, 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

Fgf20<sup>B-Cal+</sup>, Eda<sup>+/-</sup>, K14-Eda, K14-Edar, β-Cat<sup>F/F</sup>, β-Cat<sup>Ex3</sup>, K14-cre, and Sox2-GFP mice were crossed to generate Fgf20<sup>B-Cal/ B-Cal</sup>, Eda<sup>-/-</sup>, K14-Eda.Fgf20<sup>B-Cal/ B-Cal</sup>, K14-Edar.Fgf20<sup>B-Cal/ B-Cal</sup>, K14-cre-β-Cat<sup>F/F</sup>, Fgf20<sup>B-Cal/+</sup>(β-Cat<sup>Cre</sup>), Fgf20<sup>B-Cal/+</sup>(β-Cat<sup>Cre</sup>), K14-cre-β-Cat<sup>Ex3</sup>, K14-cre-β-Cat<sup>Ex3</sup>, Fgf20<sup>B-Cal/ B-Cal</sup>, Fgf20<sup>B-Cal/ B-Cal</sup>(β-Cat<sup>Cre</sup>), Fgf20<sup>B-Cal/ B-Cal</sup>(β-Cat<sup>Cre</sup>), and K14-Eda.Sox2-GFP mouse lines [Harada et al. 1999; Dassule et al. 2000; Brault 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, Q150TScoater). 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 pGlu3 Basic [Promega]. For transfection, HEK293T cells were seeded at 3 × 10<sup>5</sup> 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<sup>-/-</sup> back skins 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 Murnsky’s fixative [National Diagnostics], washed three times in PBS, and incubated in βGal-staining solution [2 mM MgCl<sub>2</sub>, 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1 mg/ml 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 [1:200; R & D Systems], β-Catenin [1:200; R&D Systems], Lef1 [1:1000, Cell Signaling Technology], phospho-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, I InBa, Wnt10b, Fg20, 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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