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

Sung-Ho Huh,^{1,3} Katja Närhi,^{2,3} Päivi H. Lindfors,² Otso Häärä,² Lu Yang,¹ David M. Ornitz,^{1,4} and Marja L. Mikkola^{2,4}

¹Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri 63110, USA;

²Developmental Biology Program, Institute of Biotechnology, University of Helsinki, 00014 Helsinki, Finland

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.

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Reciprocal mesenchymal and epithelial interactions are essential for the development of skin appendages, such as teeth, mammary glands, and hair (Schmidt-Ullrich and Paus 2005; Mikkola and Millar 2006; Fuchs 2007). Induction of hair follicle development is marked by reorganization of basal keratinocytes into a placode and clustering of underlying mesenchymal cells into a condensation, the precursor of the dermal papilla. The earliest known event in hair follicle development involves activation of epithelial *Wnt*/β-Catenin signaling: Its absence prevents (Zhang et al. 2009), and its forced activation advances (Närhi et al. 2008; Zhang et al. 2008), hair follicle induction. Subsequently, epithelial ectodysplasin (*Eda*)/*Edar* signaling is required to refine and maintain the pattern of *Wnt*/β-Catenin activity to enable primary hair placode formation (Mou et al. 2006; Fliniaux et al. 2008; Zhang et al. 2009). Pioneering tissue recombination experiments suggest that induction of hair follicles depends

on unknown cues originating from the dermis that are required for the formation of placodes (Kollar 1970; Dhouailly 1973; Hardy 1992). It is thought that a placode-derived signal subsequently induces condensation of underlying dermal cells, but the identity of this signal has been elusive (Dhouailly 1973). Although induction and patterning of hair follicles are tightly coupled (Andl et al. 2002; Sick et al. 2006; Zhang et al. 2009; Sennett and Rendl 2012), it is not known whether mesenchymal condensation or a mesenchymal prepattern is necessary for hair placode patterning.

Fibroblast growth factor (*Fgf*) signals function during multiple stages of hair follicle development. *Fgf receptor 2b* (*Fgfr2b*) is expressed in the epithelium of developing skin, and deletion of *Fgfr2b* resulted in fewer and retarded hair follicle formation (Petiot et al. 2003; Richardson et al. 2009). In addition, deletion of both *Fgfr1* and *Fgfr2* in keratinocytes resulted in progressive loss of hair follicles in adult mice (Yang et al. 2010). *Fgf10* is required for whisker formation, whereas *Fgf7* is suggested to inhibit hair follicle induction (Ohuchi et al. 2003; Richardson et al. 2009).

Here, we identify an essential role for *Fgf20* in the induction of dermal condensations in primary and secondary

³These authors contributed equally to this work.

⁴Corresponding authors

E-mail dornitz@wustl.edu

E-mail marja.mikkola@helsinki.fi

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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 (*Fgf20*^{βGal}) (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*^{βGal} 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*^{βGal} 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*^{βGal/+} background and stained for βGal activity. Activation of *Eda* in epidermis (*K14-Eda* and *K14-Edar*) resulted in increased *Fgf20*^{βGal} activity, while loss of *Eda/Edar* signaling (*Eda*^{-/-}) resulted in decreased *Fgf20*^{βGal} 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*^{ΔEx3} allele) or inhibition (conditional inactivation of β-Catenin using *K14-cre*) of epithelial Wnt/β-Catenin signaling resulted in gain and loss of *Fgf20*^{βGal} 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, 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*^{βGal/βGal} mice. Of the four morphologically distinct hair types, guard hairs were missing in *Fgf20*^{βGal/βGal} 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*^{βGal/+} and *Fgf20*^{βGal/βGal} mice were quantified. In heterozygous *Fgf20*^{βGal/+} mice, guard hairs represented 2.2% ± 1.1% of the total population. In *Fgf20*^{βGal/βGal} 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*^{βGal/+} mice to 1.8% ± 1.9% and 2.8% ± 1.7%, respectively, in *Fgf20*^{βGal/βGal} 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*^{βGal/+} mice to 95.4% ± 3.5% in *Fgf20*^{βGal/βGal} 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*^{βGal/βGal} embryos was histologically indistinguishable from *Fgf20*^{βGal/+} embryos (data not shown). At E14.5, scanning electron microscope analysis suggested the absence of primary hair follicle primordia in *Fgf20*^{βGal/βGal} embryos (Fig. 2D), yet epithelial thickenings (placodes)

Figure 1. *Fgf20* is a target of *Eda/Edar* and Wnt/β-Catenin signal during hair placode formation. (A) Real-time quantitative PCR (qPCR) for *Fgf20* from E14.5 *Eda*^{-/-} skin explants with or without EDA treatment for 2 h (*n* = 12; *P* = 0.008) or 4 h (*n* = 6; *P* = 0.03). Data are shown as mean ± SD. (B) βGal staining indicating *Fgf20*^{βGal} expression in *K14-Eda;Fgf20*^{βGal/+} and *Eda*^{-/-}; *Fgf20*^{βGal/+} embryos. (C) Assay showing that a 5.2-kb genomic DNA fragment upstream of the *Fgf20* transcription initiation site driving luciferase was induced by transfection of a β-Catenin-Lef1 fusion protein (*n* = 9; *P* < 0.001). The vector control pGL3 showed no induction. Data are shown as mean ± SD. (D) βGal staining indicating *Fgf20*^{βGal} expression in *K14-Cre;β-Cat*^{ΔEx3}(*βCat*^{act}); *Fgf20*^{βGal/+} and *K14-Cre;β-Cat*^{F/F}(*βCat*^{CKO}); *Fgf20*^{βGal/+} embryos.

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*^{βGal/+} mouse (arrow, left) but not in a *Fgf20*^{βGal/βGal} mouse (right). (B) Image of hairs from back skin of a *Fgf20*^{βGal/+} (left) or *Fgf20*^{βGal/βGal} (right) mouse. (C) Quantification of hair types from 3-wk-old mice. *Fgf20*^{βGal/βGal} 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*^{βGal/+} embryo, but the surface of a *Fgf20*^{βGal/βGal} embryo appears flat. (E) Histology of E14.5, E15.5, E16.5, and E18.5 skin from *Fgf20*^{βGal/+} and *Fgf20*^{βGal/βGal} embryos. Arrows indicate dermal condensations. (Bottom right panel) Note the bifurcated hair follicle. (au) Auchene; (g) guard; (aw) awl; (z) zigzag. Bar, 100 μm.

were histologically evident in both *Fgf20*^{βGal/+} and *Fgf20*^{βGal/βGal} embryos (Fig. 2E). Strikingly, there was no histological evidence of dermal condensation formation in *Fgf20*^{βGal/βGal} embryos (Fig. 2E). At E15.5, two types of hair follicles could be identified in *Fgf20*^{βGal/βGal} 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*^{βGal/+} embryos, the primary hair follicles reached the peg stage, and secondary hair placodes were formed. In contrast, in *Fgf20*^{βGal/βGal} 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*^{βGal/βGal} 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* (InhibinβA, previously known as activinβA), 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*^{βGal/+} embryos (Fig. 3A, left). In the absence of *Fgf20*, dermal 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*^{βGal/+} and *Fgf20*^{βGal/βGal} embryos (Supplemental Fig. S3A,B), and, occasionally, P-Cadherin⁺ foci were associated with Sox2⁺ cells in *Fgf20*^{βGal/+} embryos but not in *Fgf20*^{βGal/βGal} embryos (Supplemental Fig. S3A). At E14.0 and E14.5, Sox2 expression was readily observed in dermal condensations in *Fgf20*^{βGal/+} embryos. However, no Sox2 expression was detected in *Fgf20*^{βGal/βGal} 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*^{βGal/βGal} dermis at E13.75–E14.5 (Fig. 3F–I; Supple-

Figure 3. *Fgf20* is required for dermal condensation development. (A) Phospho-Erk1/2 staining showing loss of Erk1/2 activity in dermis from *Fgf20*^{βGal/βGal} skin (left) compared with control (right). (B–E) Coimmunostaining for Sox2 and P-Cadherin (Pcad) showing Sox2⁺ dermal condensations at E14 (B), E14.5 (C), E15.5 (D), and E16.5 (E) in *Fgf20*^{βGal/+} (left) but not in *Fgf20*^{βGal/βGal} (right) embryo skin. (F–I) In situ hybridization of *Bmp4* (F), *p21* (G), *Dkk1* (H), and *Inhba* (I) showing normal dermal expression in *Fgf20*^{βGal/+} (left) embryos and loss of expression in *Fgf20*^{βGal/βGal} embryos (right). Bar, 100 μm.

mental Fig. S3C,D). Quantitation of *Bmp4*⁺ foci showed an absence of primary dermal condensations in *Fgf20*^{βGal/βGal} embryos (Supplemental Fig. S3E). We conclude that in the absence of *Fgf20*, primary hair placodes were devoid of any known morphological and molecular evidence of dermal condensation formation.

Next, we examined dermal condensation formation and maturation at later stages of development. At E16.5, *Sox2* and *Bmp4* were expressed in primary and secondary follicles of control embryos, while *Fgf20*^{βGal/βGal} embryos showed rare *Sox2*⁺ cells in the few follicles that had advanced further (Fig. 3E; Supplemental Fig. S4A,B).

However, the majority of *Fgf20*^{βGal/βGal} hair follicles remained at the placode/germ stage and were negative for all condensation markers analyzed (Fig. 3E; Supplemental Fig. S4A). *Corin*, a marker of maturing dermal papillae of all hair types (Enshell-Seijffers et al. 2008), was also absent from *Fgf20*^{βGal/βGal} embryos but was readily detected in primary follicles of control embryos at E16.5 (Supplemental Fig. S4B). At E17.5, *Sox2* and *Corin* were expressed in both primary and secondary follicles of *Fgf20*^{βGal/+} embryos, while in *Fgf20*^{βGal/βGal} skin, many (secondary) follicles showed an absence or low level of expression of both markers (Supplemental Fig. S4C). *CD133*, another marker of dermal condensations (Ito et al. 2007), was expressed at E15.5 and E16.5 (Supplemental Fig. S4D,E, left) in *Fgf20*^{βGal/+} embryos. However, *CD133* expression was not detected in *Fgf20*^{βGal/βGal} embryos at the same time point (Supplemental Fig. S4D,E, right). Together, these data indicate that *Fgf20* is critical for formation of primary dermal condensations and that dermal condensations of secondary follicles were also affected in *Fgf20*^{βGal/βGal} embryos, but to a lesser extent.

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

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*^{βGal/βGal} 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*^{βGal/βGal} embryos (Fig. 4A–C). Also, *Fgf20*^{βGal} activity showed a similar pattern in *Fgf20*^{βGal/βGal} embryos, in contrast to the punctuate expression seen in *Fgf20*^{βGal/+} 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*^{βGal/βGal} 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*^{βGal/βGal} embryos (Fig. 4F). Furthermore, nuclear *Lef1* protein was reduced in the dermis of *Fgf20*^{βGal/βGal} 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

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*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos. (D) *Fgf20*^{βGal} staining showing increased interfollicular *βGal* staining in *Fgf20*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos. (E) In situ hybridization of *Axin2* showing increased expression in placodal cells but decreased expression in dermal cells in *Fgf20*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos. (F) Immunostaining for *β-Catenin* showing loss of *β-Catenin* nuclear localization (arrows) in dermal cells in *Fgf20*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos. (G) Immunostaining for *Lef1* shows strong nuclear expression in the dermal condensation of *Fgf20*^{βGal/+} but not in *Fgf20*^{βGal/βGal} embryos at E14.5. (H,I) In situ hybridization for *Dkk4* (H) and *Sostdc1* (I) showing severely reduced expression in *Fgf20*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} 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*^{*βGal/βGal*} 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*^{*βGal/βGal*} embryos, may explain the observed phenomenon.

We next analyzed expression of *Edar* and observed severely decreased levels in *Fgf20*^{*βGal/βGal*} embryos compared with *Fgf20*^{*βGal/+*} embryos (Fig. 5A). *Ik-Bα*, the downstream target of *Eda/Edar* (Schmidt-Ullrich et al. 2006; Pummila et al. 2007), was similarly decreased in *Fgf20*^{*βGal/βGal*} 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.

Figure 5. *Fgf20* modulates *Eda/Edar* but not *Shh* signaling during primary hair placode formation. (A–B) In situ hybridization for *Edar* (A) and *Ik-Bα* (B) showing decreased expression in *Fgf20*^{*βGal/βGal*} embryos compared with *Fgf20*^{*βGal/+*} embryos. (C,D) In situ hybridization of *Shh* (C) and *Patched1* (*Ptch*) (D) showing unaltered expression in *Fgf20*^{*βGal/βGal*} embryos compared with *Fgf20*^{*βGal/+*} embryos. Bar, 100 μ m.

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*^{*βGal/βGal*} 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*^{*βGal/βGal*} embryos.

We also noted that in *Fgf20*^{*βGal/βGal*} embryos, *Shh* expression was confined to placodal cells, unlike *Wnt10b* and β Gal (*Fgf20*^{*βGal*}), 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; Pummila et al. 2007). In *Fgf20*^{*βGal/βGal*} embryos, *Eda/Edar* signaling is greatly reduced (Fig. 5A,B). To address whether *Shh* expression and placode formation in *Fgf20*^{*βGal/βGal*} embryos requires *Eda*, we generated mice deficient in both *Fgf20* and *Eda*. *Eda*^{*-/-*}; *Fgf20*^{*βGal/+*} and *Eda*^{*-/-*}; *Fgf20*^{*βGal/βGal*} embryos lacked all expression of *Shh* at E14.5 (Supplemental Fig. S5C,D). These data indicate that *Edar* signaling, although strongly reduced, is nevertheless necessary for *Shh* expression in *Fgf20*^{*βGal/βGal*} embryos. We propose that the relatively normal patterning of *Shh* expression and morphological placodes in *Fgf20* mutants is achieved via simultaneous activation of *Edar* and Wnt/ β -Catenin pathways.

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-Cat*^{*ΔEx3*}) embryos. We addressed the signaling hierarchy between *Fgf20* and *Eda* by comparing *Fgf20*^{*βGal/+*} and *Fgf20*^{*βGal/βGal*} embryos with *K14-Eda;Fgf20*^{*βGal/+*} and *K14-Eda;Fgf20*^{*βGal/βGal*} 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 (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*^{*βGal/βGal*} 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 *Fgf20*^{*βGal/βGal*} 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.

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*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos at E14.5. (B) In situ hybridization for *Ik-Bα* showing increased expression in *K14-Eda* and *K14-Eda;Fgf20*^{βGal/βGal} 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*^{ΔEx3} (*βCat*^{act}) were absent in *Fgf20*^{βGal/βGal} embryos compared with *Fgf20*^{βGal/+} embryos, yet premature induction of placodes was unaffected by loss of *Fgf20* (cf. Supplemental Fig. S1A). Bar, 100 μm.

To determine whether epidermal Wnt/β-Catenin signaling could rescue dermal condensation formation in the absence of *Fgf20*, we compared *Fgf20*^{βGal/+} and *Fgf20*^{βGal/βGal} embryos with *K14-Cre;β-Cat*^{ΔEx3}; *Fgf20*^{βGal/+} and *K14-Cre;β-Cat*^{ΔEx3}; *Fgf20*^{βGal/βGal} embryos. Forced activation of epidermal Wnt/β-Catenin signaling in *Fgf20*^{βGal/+} embryos resulted in ectopic dermal condensation formation, as shown by expanded Sox2 expression at E13.5 (cf. Figs. 3B, 6A and 6C). However, in *K14-Cre;β-Cat*^{ΔEx3}; *Fgf20*^{βGal/βGal} embryos, dermal condensations failed to form, and Sox2 expression was not detected (Fig. 6C, right). Despite the absence of dermal condensations in *K14-Cre;β-Cat*^{ΔEx3} embryos lacking *Fgf20*, hair placodes were precociously induced, as evidenced by P-Cadherin expression (cf. Fig. 6C and Supplemental Fig. S1A). Together, these data indicate that *Fgf20* is not required for placode formation but functions downstream from Eda/Edar and Wnt/β-Catenin signaling to induce formation of the dermal condensations, without which guard hairs cannot form.

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 downgrowth 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 prepatterning 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*^{βGal}) 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 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

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 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- κ B 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

Fgf20 ^{β Gal/+}, *Eda*^{+/+}, *K14-Eda*, *K14-Edar*, β -Cat^{F/F}, β -Cat ^{Δ Ex3}, *K14-cre*, and *Sox2-GFP* mice were crossed to generate *Fgf20* ^{β Gal/ β Gal}, *Eda*^{+/+}, *K14-Eda*;Fgf20 ^{β Gal/ β Gal}, *K14-Edar*;Fgf20 ^{β Gal/ β Gal}, *K14-cre*; β -Cat^{F/F};Fgf20 ^{β Gal/+} [β -Cat^{CKO};Fgf20 ^{β Gal/+}], *K14-cre*; β -Cat ^{Δ Ex3}; Fgf20 ^{β Gal/+} [β -Cat^{act};Fgf20 ^{β Gal/+}], *K14-cre*; β -Cat ^{Δ Ex3}; Fgf20 ^{β Gal/ β Gal} [β -Cat^{CKO};Fgf20 ^{β Gal/ β Gal}], and *K14-Eda*;Sox2-GFP mouse lines (Harada et al. 1999; Dassule et al. 2000; Brault et al. 2001; Laurikkala 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 3×10^5 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 (Fliniaux et al. 2008). Three independent experiments with triplicate samples were performed.

Quantitative RT-PCR (qRT-PCR)

E14.5 *Eda*^{+/+} 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- μ L 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 (Fliniaux 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*.

β Gal staining

Embryos at various stages of development were fixed overnight in Mirsky's fixative (National Diagnostics), washed three times in PBS, and incubated in β Gal-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 β Gal-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 Histo-resin as specified by the manufacturer (Leica) and stained with haematoxylin and eosin.

Immunohistochemistry

Embryos were embedded in OCT, frozen, and cryosectioned at 12 μ m. Alternatively, paraffin-embedded tissues were serially sectioned at 7 μ m, 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: β Gal (1:500; abcam), Sox2 (1:500 [Millipore] or 1:200 [Santa Cruz Biotechnology]), P-Cadherin (1:100 [gift from Dr. Takeichi] or 1:50 [R&D Systems]), Corin (1:800; gift from Dr. Morgan), 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*, *IkB α* , *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 35 S-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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Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles

Sung-Ho Huh, Katja Närhi, Päivi H. Lindfors, et al.

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