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WNT Signals Are Required for the Initiation of Hair Follicle Development

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Summary

Hair follicle morphogenesis is initiated by a dermal signal that induces the development of placodes in the overlying epithelium. To determine whether WNT signals are required for initiation of follicular development, we ectopically expressed Dickkopf 1, a potent diffusible inhibitor of WNT action, in the skin of transgenic mice. This produced a complete failure of placode formation prior to morphological or molecular signs of differentiation, and blocked tooth and mammary gland development before the bud stage. This phenotype indicates that activation of WNT signaling in the skin precedes, and is required for, localized expression of regulatory genes and initiation of hair follicle placode formation.

Introduction

Hair follicles contain two major types of cell: dermal papilla cells, derived from the mesenchyme, and epithelial cells of the root sheaths and hair shaft, originally derived from the surface epithelium (Hardy, 1992). The formation of hair and feather follicles during embryogenesis requires a series of reciprocal inductive interactions between the epithelium and mesenchyme, which are initiated by a "first dermal message" from the mesenchyme that causes the formation of placodes, or thickenings, in the surface epithelium (Hardy, 1992). The first dermal message is thought to trigger the activation of promoters and repressors of follicle fate that compete with each other to establish a regular array of placodes (reviewed in Millar, 2002).

Once placodes are formed, they send an epithelial message to the mesenchyme causing the clustering of mesenchymal cells to form a dermal condensate, which later develops into the hair follicle dermal papilla (Hardy, 1992). A second dermal message from the dermal condensate to the epithelium causes the proliferation of epithelial cells and their downgrowth into the dermis (Hardy, 1992).

Members of several families of secreted signaling molecules are expressed in developing hair and feather follicles, and some of these have been shown to play important roles in communication between epithelial and mesenchymal cells (reviewed in Millar, 2002). However, several key signals required for early steps in hair follicle formation remain unidentified or incompletely characterized. In particular, the signals that regulate production of the first dermal message, the identity of the first dermal message, and the mechanisms by which the epithelium initiates its response to this message are unknown (Millar, 2002).

WNT paracrine signaling molecules play essential roles in many aspects of development and tumorigenesis (reviewed in Wodarz and Nusse, 1998). Binding of WNT proteins to their receptors, Frizzled (FZ) proteins, and to essential coreceptors of the low-density lipoprotein receptor-related protein (LRP) family activates a conserved "canonical" signaling pathway that causes stabilization of cytoplasmic β-catenin, its translocation to the nucleus, and the formation of active transcription complexes between β-catenin and members of the LEF/TCF family of DNA binding factors (Bejsovec, 2000; Wodarz and Nusse, 1998). WNTs also signal through alternate pathways involving Frizzled receptors, but not LRPs, β-catenin, or LEF/TCF factors (Niehrs, 2001).

Nuclear localization of β-catenin is observed in the dermis underlying the chick feather tract 2 days before the appearance of placodes (Noramly et al., 1999), and expression of *Lef1* in the dermis is necessary for the initiation of mouse vibrissa, but not pelage, follicle formation (Kratochwil et al., 1996), suggesting that WNT signaling may be involved in activating the first dermal message for at least some types of follicle. Consistent with this hypothesis, WNT1 can substitute for signals from the dorsal neural tube that are required for the development of dorsal, feather-inducing dermis in the chick (Olivera-Martinez et al., 2001).

Activation of the canonical WNT signaling pathway may also be necessary for subsequent steps in follicle development, as *Lef1* is required in the epithelium for completion of vibrissa follicle development and for early stages in the development of most pelage hairs in mice (Kratochwil et al., 1996). Progressive, Cre-mediated deletion of the β-*catenin* gene in mouse epidermis also causes failure of placode morphogenesis, although very early molecular markers of placodes are expressed in a localized fashion (Huelsken et al., 2001). A WNT-responsive TOPGAL reporter gene, and several *Wnt* genes, are expressed in the placodes and dermal condensates of developing hair follicles (DasGupta and Fuchs, 1999; Reddy et al., 2001), consistent with the hypothesis that WNT signals are important for crosstalk between the epithelium and mesenchyme during follicle development.

These data suggest that activation of the WNT signaling pathway may be required for generation of the first dermal message in chick dorsum, for initiation of formation of vibrissa follicles, and for early stages in the development of most pelage hair follicles. However, several key questions remain unanswered. First, it is not clear whether activation of the WNT signaling pathway is universally required for initiation of hair follicle development, or is only necessary for initiating the formation of certain subtypes of follicle. Second, there are currently no reports of hair follicle phenotypes in mice carrying loss-of-function mutations in *Wnt* genes, and evidence

development stems mainly from the phenotypes of mice carrying gain- and loss-of-function mutations in downstream effector genes of the canonical WNT signaling pathway, such as β-*catenin* and *Lef1* (Gat et al., 1998; Huelsken et al., 2001; Kratochwil et al., 1996; Zhou et al., 1995). Indeed, since β-*catenin* and *Lef1* mRNAs are elevated in placodes (Huelsken et al., 2001; Widelitz et al., 2000; Zhou et al., 1995), and WNT signaling has classically been shown to regulate β-*catenin* at the level of protein stability rather than transcription (Wodarz and Nusse, 1998), it has been suggested that the functions of β-*catenin* in developing follicles may be governed by mechanisms that are independent of WNT-WNT receptor interactions (Huelsken et al., 2001). Whether such interactions are required therefore remains an open question.

To test the hypothesis that WNT proteins are required for initiating hair follicle development, we generated transgenic mice ectopically expressing Dickkopf 1 (DKK1), a potent and specific endogenous secreted inhibitor of WNT action (Bafico et al., 2001; Glinka et al., 1998; Mao et al., 2001; Semenov et al., 2001; Zorn, 2001), in basal cells of the epidermis. DKK1 functions by binding and inhibiting LRP coreceptors required for activation of canonical WNT signaling (Bafico et al., 2001; Glinka et al., 1998; Mao et al., 2001; Semenov et al., 2001; Zorn, 2001), and is diffusible in vivo (Glinka et al., 1998; Zorn, 2001). Thus, DKK1 protein ectopically expressed in basal epidermal cells is likely to be capable of blocking canonical WNT signaling in adjacent dermal cells as well as in the epidermis.

We show that mice expressing high levels of *Dkk1* in the skin display an early and complete block in the development of skin appendages including all types of hair follicle, vibrissae, teeth, and mammary glands. Our results demonstrate that the actions of WNT proteins are required for the initiation of hair follicle development and for formation of tooth and mammary gland buds. We find that expression of *Dkk1* prevents localized expression of all hair follicle placode markers tested, indicating that WNT signals precede and are required for activation of other regulators of hair follicle development.

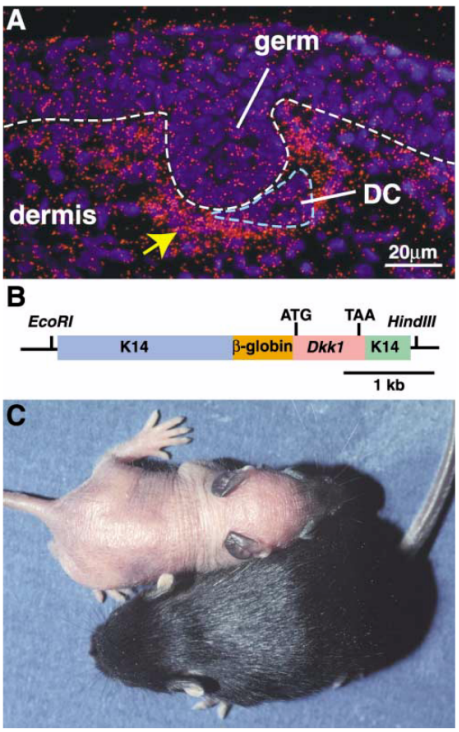

Figure 1. Endogenous Expression Pattern of *Dkk1* mRNA, K14-*Dkk1* Transgene, and Postnatal Phenotype of a Transgenic Mouse

(A) In situ hybridization of sectioned embryonic mouse skin at E15.5 with a ^{35}S-labeled probe for *Dkk1*. The dermal-epithelial border is indicated by a dashed white line. A germ stage follicle is indicated. The dermal condensate of the hair follicle is outlined by a dashed blue line. Hybridization appears as red grains and is indicated by a yellow arrow, and nuclei are counterstained with Hoechst and appear blue. *Dkk1* expression is elevated in the dermis surrounding the follicle but is excluded from the follicle itself. DC, dermal condensate.

(B) Transgene used for ectopic expression of *Dkk1* cDNA. The transgene includes a rabbit β-globin intron for transcript stability and promoter and polyA addition sequences from the human K14 gene.

(C) Phenotype of F1 progeny of K14-*Dkk1* founder #7 at postnatal day 16. The mouse at the top of the photograph expresses intermediate to high levels of the transgene, determined by in situ hybridization of *Dkk1* probe to a sectioned skin biopsy, and is almost completely hairless. The mouse below it is a nontransgenic littermate.

Results

Endogenous *Dkk1* Is Expressed in the Mesenchyme Surrounding Developing Hair Follicles

To determine the endogenous expression pattern of *Dkk1* in developing wild-type mouse skin, we carried out in situ hybridization for *Dkk1* on sagittal sections of embryos between embryonic day (E) 14.5 and E18.5. At E14.5 and E15.5, *Dkk1* is expressed at low levels in the dermis and is specifically upregulated in the mesenchyme surrounding developing hair follicles at the placode and germ stages, but is excluded from the hair follicles themselves (Figure 1A). At E16.5 and E18.5, *Dkk1* expression continues in dermal cells surrounding the follicles and also localizes to a subset of epithelial cells in follicles at more advanced stages of develop-

Ectopic Expression of *Dkk1* in the Epidermis Causes Defects in Vibrissae, Hair, Teeth, and Mammary Glands

To determine whether canonical WNT signals are required for the development of hair follicles, we placed the *Dkk1* cDNA under the control of a keratin (K) 14 promoter (Wang et al., 1997; Figure 1B), which directs expression to surface epithelium starting at E9.5 (Byrne et al., 1994) and to basal cells of the epidermis on stratification (Wang et al., 1997). Founder mice were produced by pronuclear injection of the transgene into fertilized eggs. The location and levels of transgene expression were determined by in situ hybridization of sectioned embryos or skin biopsies with a probe for *Dkk1*. In transgenic founder embryos, the transgene was expressed

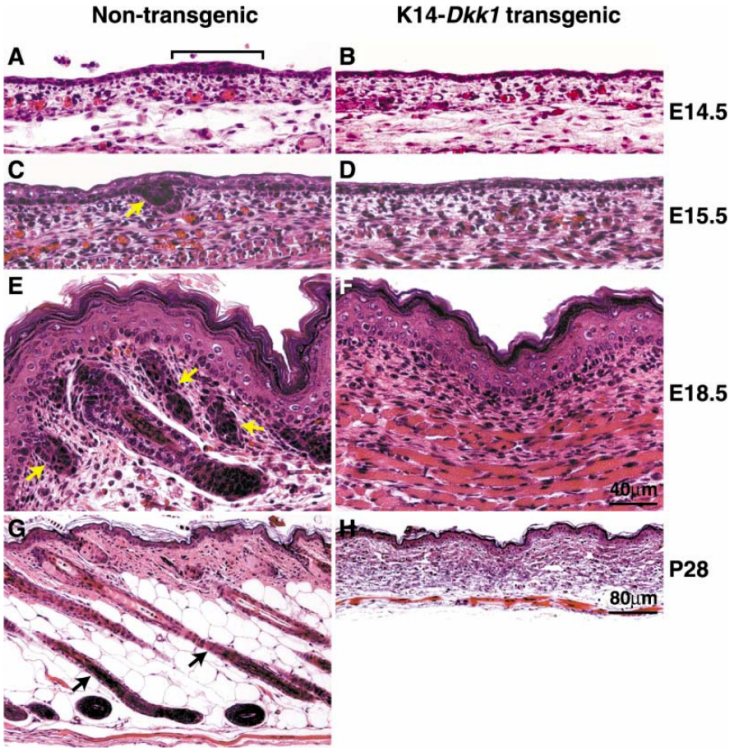

Figure 2. K14-*Dkk1* Transgenic Skin Lacks Hair Follicles

Sections of nontransgenic littermate (A, C, E, and G) and high-expressing K14-*Dkk1* transgenic (B, D, F, and H) skin at E14.5 (A and B), E15.5 (C and D), E18.5 (E and F), and postnatal day 28 (G and H), stained with hematoxylin and eosin. Sections shown in (A)–(F) are from transgenic founder embryos and their littermates. Sections shown in (G) and (H) are of ventral skin from F1 progeny of founder #7. Primary hair follicle development in nontransgenic skin begins with the formation of placodes (bracketed in [A]), which develop into germ stage follicles (indicated by a yellow arrow in [C]). Development of secondary follicles at E18.5 is indicated by yellow arrows in (E). Mature anagen stage follicles are present in ventral skin at P28 ([G], black arrows). All stages of development of both primary and secondary hair follicles are absent from high-expressing transgenic skin. (A)–(F) were photographed at 20× magnification; (G) and (H) were photographed at 10× magnification.

tongue epithelium, consistent with previous reports of K14 promoter activity (Byrne et al., 1994; Wang et al., 1997). Mice expressing high levels of the transgene were born with open eyes and lacking vibrissae, and died soon after birth. Mice expressing lower levels of the transgene, or mosaic for transgene expression, were viable and displayed sparse hair or patches of absent hair. F1 progeny produced by one of the mosaic founder animals (#7) expressed intermediate to high levels of the transgene and lived for up to 60 days. These mice lacked teeth, were completely hairless on the ventrum, had extremely sparse hair on the dorsum (Figure 1C), and the females lacked external signs of mammary gland development.

K14-*Dkk1* Transgenic Mice Lack Hair Follicles

In mice, formation of vibrissa follicles begins at E12. In contrast, the development of pelage hair follicles occurs in several waves, with formation of large primary hair follicles that give rise to guard hairs being initiated at E14.5 in the dorsum and slightly later in the ventrum, and formation of smaller, secondary follicles that produce awl and zigzag hairs being initiated between E17 and birth (Mann, 1962). To determine the basis of the hair phenotype of transgenic mice, we performed histological analysis on transgenic founders and nontransgenic littermates sacrificed at E14.5, E15.5, E18.5, postnatal day (P) 1 and P17, and F1 progeny of a mosaic transgenic founder (#7) sacrificed at P13, P17, P28, P33, and P52. In nontransgenic embryos, the normal sequence of hair follicle development was observed (Figures 2A, 2C, and 2E). In contrast, littermate embryos showed no sign of development of either primary or secondary hair follicles at any stage analyzed (Figures 2B, 2D, and 2F), and vibrissa follicles were either entirely absent or very much reduced in number.

Founder embryos showing weaker hybridization of *Dkk1* probe to the epidermis, indicating low to intermediate levels of transgene expression, displayed marked reductions in the numbers of hair follicles compared with nontransgenic littermate embryos, with secondary hair follicles being most affected. Postnatal progeny of founder #7 showed a complete absence of hair follicles on the ventrum (Figure 2H) and sparse guard hair follicles on the dorsum at P13, P17, P28, P33, and P52. The ventral dermis appeared very thin, in part due to the absence of hair follicles (compare Figures 2G and 2H). Dermal fat was reduced in transgenic ventral and dorsal skin, possibly as a consequence of poor nutrition caused by the absence of teeth. The differential effects of *Dkk1* expression on dorsal and ventral, and primary and secondary, hair follicle development in mice expressing low and intermediate levels of the transgene correlate with expression driven by the K14 promoter, which increases between E9.5 and birth (Byrne et al., 1994). Our results indicate that the development of vibrissa follicles and all types of hair follicle is completely blocked by high levels of ectopic DKK1.

Ectopic *Dkk1* Does Not Affect Differentiation or Proliferation of the Epidermis

Despite the dramatic effects of *Dkk1* on hair follicle formation, differentiation of the epidermis in embryos and postnatal mice expressing high levels of *Dkk1* appeared

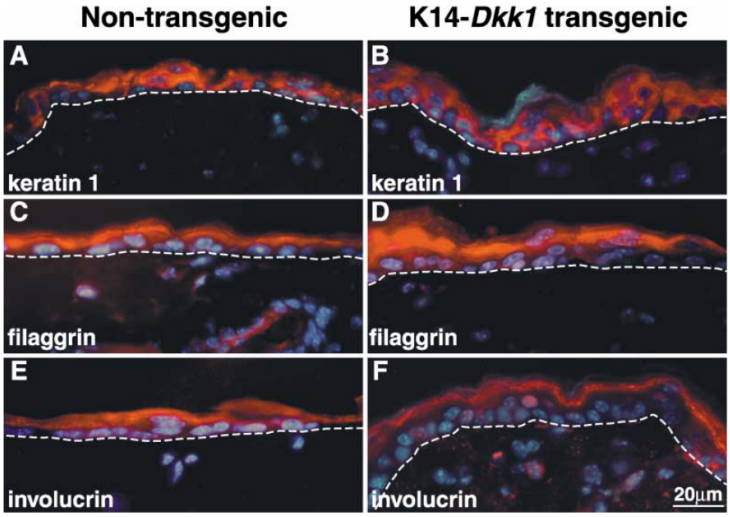

Figure 3. Expression of the K14-*Dkk1* Transgene Does Not Affect Expression of Epidermal Differentiation Markers

Immunofluorescence of sections of ventral skin from transgenic mice of line #7 (B, D, and F) and nontransgenic littermates (A, C, and E) at P28 with antibodies to the proteins indicated. The dermal-epidermal junction is indicated by a dashed white line in each panel.

whether *Dkk1* affects epidermal differentiation at the molecular level, we used immunofluorescence to assay for expression of differentiation markers including K1 and K10, which are normally expressed in the spinous layers of the epidermis, and involucrin, filaggrin, and loricrin, which are expressed in more superficial layers. Analyses were carried out at E18.5 on transgenic founder embryo skin expressing high levels of K14-*Dkk1*, and at postnatal days 13 and 28 on hairless ventral skin of transgenic mice from line #7, and compared with results using nontransgenic littermate skin. The patterns of expression of differentiation markers were indistinguishable in transgenic and control skin at all stages examined (Figures 3A–3F and data not shown). Therefore, ectopic expression of *Dkk1* specifically affects hair follicle differentiation in the skin.

Analysis of the expression patterns of K6 and K17, which are markers for hair follicles and hyperproliferative epidermis, and assays for BrdU incorporation in dorsal and ventral skin from transgenic and nontransgenic littermate mice of line #7 at postnatal days 32 and 52, showed no significant differences in the proliferative status of transgenic and nontransgenic epidermis (data not shown). These results contrast with those reported for progressive loss of epidermal β-catenin, which causes an approximately 10-fold increase in proliferation (Huelsken et al., 2001), possibly due to the fact that, in the latter case, degenerating hair follicles are present in the skin.

Ectopic *Dkk1* Blocks Patterned Upregulation of *Wnt10b* in the Epithelium

Of several *Wnt* genes expressed in developing hair follicles, *Wnt10b* shows the earliest and most marked localization to placodes (Reddy et al., 2001). *Wnt10b* causes mammary tumors in mice, stabilization of β-catenin in preadipocytes, and partial axis duplication in *Xenopus* embryos, indicating that it is capable of directing the canonical signaling pathway, and is therefore a strong candidate for the signal that activates canonical signal

and references therein). If the phenotype of K14-*Dkk1* mice is solely due to inhibition of WNT10b function, we would expect expression of the *Wnt10b* gene to be unaffected by the transgene. We therefore examined *Wnt10b* expression in transgenic skin. In nontransgenic control embryos, *Wnt10b* was expressed at low levels in the interfollicular epidermis, showed markedly elevated patterned expression in the epithelium at sites of developing placode and germ stage hair follicles at E14.5 and E15.5 (Figure 4C and data not shown), and was expressed in the epithelial compartment of follicles analyzed at later stages. However, in littermates expressing high levels of K14-*Dkk1* transgene (Figures 4B and 4D), *Wnt10b* was expressed at low levels in the epidermis, but we found no evidence for patterned elevated expression of this gene at E14.5, E15.5, or E18.5 (Figure 4D and data not shown). In contrast, expression of *Wnt6* and *Fz6*, which are normally expressed uniformly in the surface epithelium and placodes, was unaffected by ectopic expression of *Dkk1* (Figures 4E and 4F and data not shown). These results suggest either that *Wnt10b* regulates its own accumulation or that *Dkk1* inhibits signals upstream of *Wnt10b* that are required for its patterned upregulation.

Patterned Upregulation of β-*catenin* mRNA in the Epithelium Is Blocked by Ectopic *Dkk1*

β-*catenin* mRNA is upregulated in the epithelium at sites of hair follicle formation, and K14-Cre-mediated deletion of the β-*catenin* gene causes failure of placode development, indicating that β-catenin plays an essential role in follicular morphogenesis (Huelsken et al., 2001). To determine whether patterned upregulation of β-*catenin* message occurs in the presence of ectopic *Dkk1*, we examined expression of β-*catenin* mRNA in high-expressing K14-*Dkk1* transgenic embryos and nontransgenic littermates at E14.5, E15.5, and E18.5. The expected pattern of expression was observed in controls (Figure 4G and data not shown); however, transgenic embryos

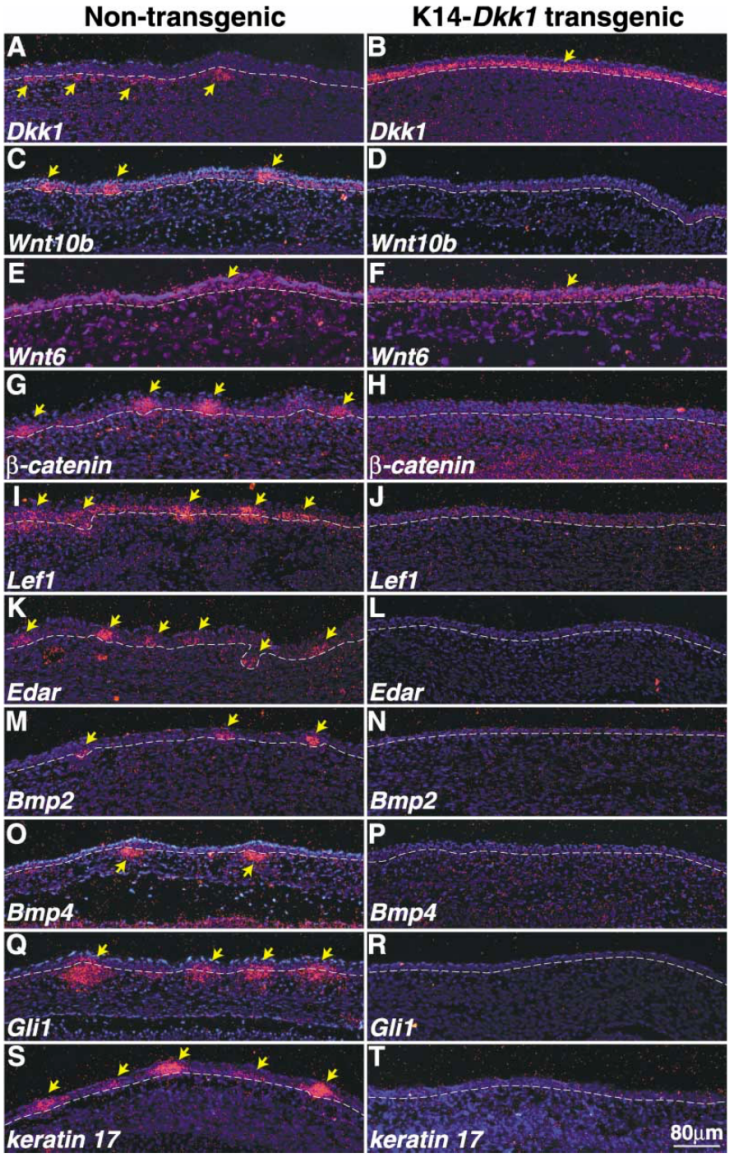

Figure 4. Patterned Expression of Hair Follicle Placode Markers Is Absent from K14-*Dkk1* Transgenic Skin

Sagittal sections of skin from nontransgenic (A, C, E, G, I, K, M, O, Q, and S) and high-expressing transgenic (B, D, F, H, J, L, N, P, R, and T) embryos at E14.5–E15.5 were hybridized with ^{35}S-labeled probes for the genes indicated. The dermal-epithelial junction is marked by a dashed white line in each panel. Hybridization is indicated by yellow arrows. Expression of endogenous *Dkk1* is seen in the dermis at sites of hair follicle initiation (A) but is absent from the dermis of mice ectopically expressing *Dkk1* in the epithelium (B). Expression of *Wnt10b*, β-*catenin*, *Lef1*, *Edar*, *Bmp2*, *Bmp4*, *Gli1*, and *K17* is observed at sites of hair follicle initiation in nontransgenic skin, but patterned upregulation of these genes is not seen in transgenic skin (C, D, and G–T). *Wnt6* is expressed throughout the epithelium in both nontransgenic and transgenic skin (E and F). All panels were photographed at the same magnification.

Elevated Expression of *Wnt10b* at Sites of Primary Hair Follicle Development Requires EDA Signaling

Mutations in the genes encoding ectodysplasin (EDA) and its receptor, ectodysplasin receptor (EDAR), block very early stages in the development of primary and zigzag hair follicles in mice, although awl hair follicles and some vibrissae are unaffected (Ferguson et al., 1997; Headon and Overbeek, 1999; Srivastava et al., 1997; Vielkind and Hardy, 1996). *Edar* is initially expressed uniformly in the surface epithelium and becomes upregulated at sites of hair follicle development (Headon and Overbeek, 1999; Zhou et al., 1995). Patterned expression of *Edar* at E15.5 is not affected by absence of epithelial β-catenin, suggesting that WNT signaling in placodes lies genetically downstream of EDA/EDAR signaling, or is regulated independently we examined *Wnt10b* expression in E14.5 and E15.5 male embryos carrying a null mutation in the X-linked *Eda* gene. Patterned expression of *Wnt10b* was observed in the truncal, limb, and head surface epithelium of wild-type littermate embryos, but was absent from male mutants (Figures 5A and 5B and data not shown). In contrast, expression of *Wnt10b* in the developing vibrissa follicles of male mutants was similar to that observed in wild-type littermates (Figures 5C and 5D). Thus, patterned expression of *Wnt10b* lies downstream of EDA/EDAR signaling in primary, but not vibrissa, follicles.

Ectopic *Dkk1* Blocks Localized Expression of *Edar* and *Lef1*

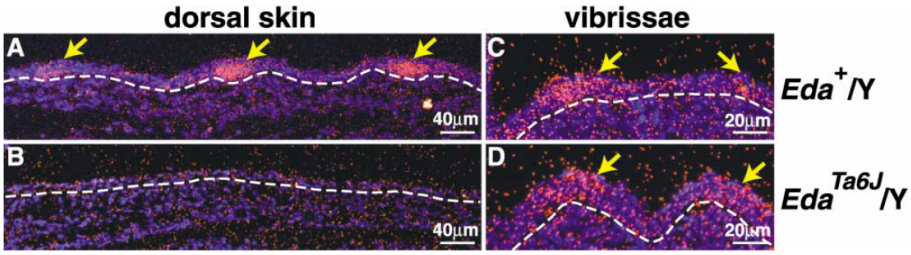

Figure 5. EDA Signaling Is Required for Localized Expression of *Wnt10b* in Dorsal Skin but Not Vibrissa Follicles

Sections of dorsal skin (A and B) and vibrissa pad (C and D) from male $Eda^{Ta\text{-}6J}$/Y (B and D) and Eda^{+}/Y littermates (A and C) at E14.5 were hybridized with a probe for *Wnt10b*. *Wnt10b* is upregulated in a patterned fashion in the dorsal skin of Eda^{+}/Y but not $Eda^{Ta\text{-}6J}$/Y embryos (A and B). Expression of *Wnt10b* in developing vibrissa follicles is unaffected by the $Eda^{Ta\text{-}6J}$ mutation (C and D). (A) and (B) were photographed at 10× magnification and (C) and (D) at 20× magnification.

Overbeek, 1999; Zhou et al., 1995), was altered by ectopic expression of *Dkk1* in the skin. Patterned upregulation of *Edar* and *Lef1* was seen in the epithelium of nontransgenic embryos at E14.5, E15.5, and E18.5, but was absent at all of these stages from the skin of embryos expressing high levels of K14-*Dkk1* transgene (Figures 4I–4L and data not shown). These data demonstrate that *Dkk1* blocks hair follicle development upstream of localized expression of *Edar* and *Lef1*. Hair follicle development is therefore blocked at an earlier stage in K14-*Dkk1* transgenic mice than in mice bearing a K14-Cre-mediated deletion of β-*catenin*, in which *Edar* and *Lef1* are expressed in a patterned fashion at E15.5 (Huelsken et al., 2001). Since localized expression of *Wnt10b* lies downstream of EDA/EDAR signaling in primary follicles (see above), our results indicate that inhibition of WNT10b signaling in placodes is not sufficient to account for the hair follicle phenotype of K14-*Dkk1* mice. Since current data indicate that DKK1 is a specific inhibitor of canonical WNT signaling (Bafico et al., 2001; Glinka et al., 1998; Mao et al., 2001; Semenov et al., 2001; Zorn, 2001), our results suggest that WNT signals upstream of localized *Wnt10b* regulate the patterned accumulation of *Edar*, *Lef1*, β-*catenin*, and *Wnt10b* mRNAs.

Ectopic *Dkk1* Blocks Expression of Additional Placode Markers and Molecules Necessary for Early Steps in Hair Follicle Development

Other regulators of early stages of hair follicle development include Sonic hedgehog (SHH), GLI1, a transcriptional effector of SHH signaling, and bone morphogenic proteins (BMPs) 2 and 4 (reviewed in Millar, 2002). Patterned expression of the genes encoding these factors and another BMP family member, BMP7, was observed in control but not transgenic embryos at E14.5, E15.5, and E18.5 (Figures 4M–4R and data not shown). These results are consistent with previous observations that *Shh* and *Bmp*s lie downstream of both EDA/EDAR signaling and epithelial β-catenin in hair follicle development (Headon and Overbeek, 1999; Huelsken et al., 2001).

Expression of *Dkk1* is elevated in the dermis at sites of placode development in control embryos (Figures 1A the dermis (Figure 4B), indicating that ectopic expression of *Dkk1* in epithelial cells directly or indirectly prevents elevated dermal expression of endogenous *Dkk1*.

K17 is a very early marker for sites where hair follicles will form, and normally shows patterned expression in the epidermis from E10.5 (McGowan and Coulombe, 1998). Remarkably, *K17* mRNA also failed to show patterned expression in the skin of embryos expressing high levels of the K14-*Dkk1* transgene at E14.5, E15.5, and E18.5 (Figures 4S and 4T and data not shown). DKK1 therefore blocks hair follicle morphogenesis at a stage prior to the activation of signaling pathways important for early stages in hair follicle development, and prior to the expression of known placode markers.

Ectopic Expression of *Dkk1* Blocks Formation of Other Skin Appendages

In addition to blocking hair follicle morphogenesis, expression of *Dkk1* in the epidermis prevents the development of other epidermal appendages that depend on epithelial-mesenchymal interactions for their formation. Histological analysis of the oral cavities of embryos expressing high levels of the K14-*Dkk1* transgene revealed that molar and incisor tooth development is blocked before the bud stage although thickening of the oral epithelium was observed, indicating that presumptive dental epithelium is formed in transgenic embryos (Figures 6A and 6B). Consistent with failure of tooth development, the mandibles of postnatal transgenic mice lacked alveolar ridges, which normally develop in response to tooth formation (Figures 6E and 6F). Similarly, histological analysis of high-expressing embryos at E14.5 revealed that mammary gland development was arrested before the bud stage (Figures 6A and 6B). Skin dissected from high-expressing embryos at E13.5 and examined under the light microscope also showed no sign of mammary bud formation (Figure 6D), although mammary buds were clearly visible in nontransgenic control skin (Figure 6C).

Eyelid fusion normally occurs at E16, with the eyelids remaining fused until P11. Late-stage embryos and newborn mice expressing high levels of ectopic *Dkk1* displayed open eyes, suggesting a role for canonical WNT signaling in regulating eyelid fusion (Figures 6G and 6H)

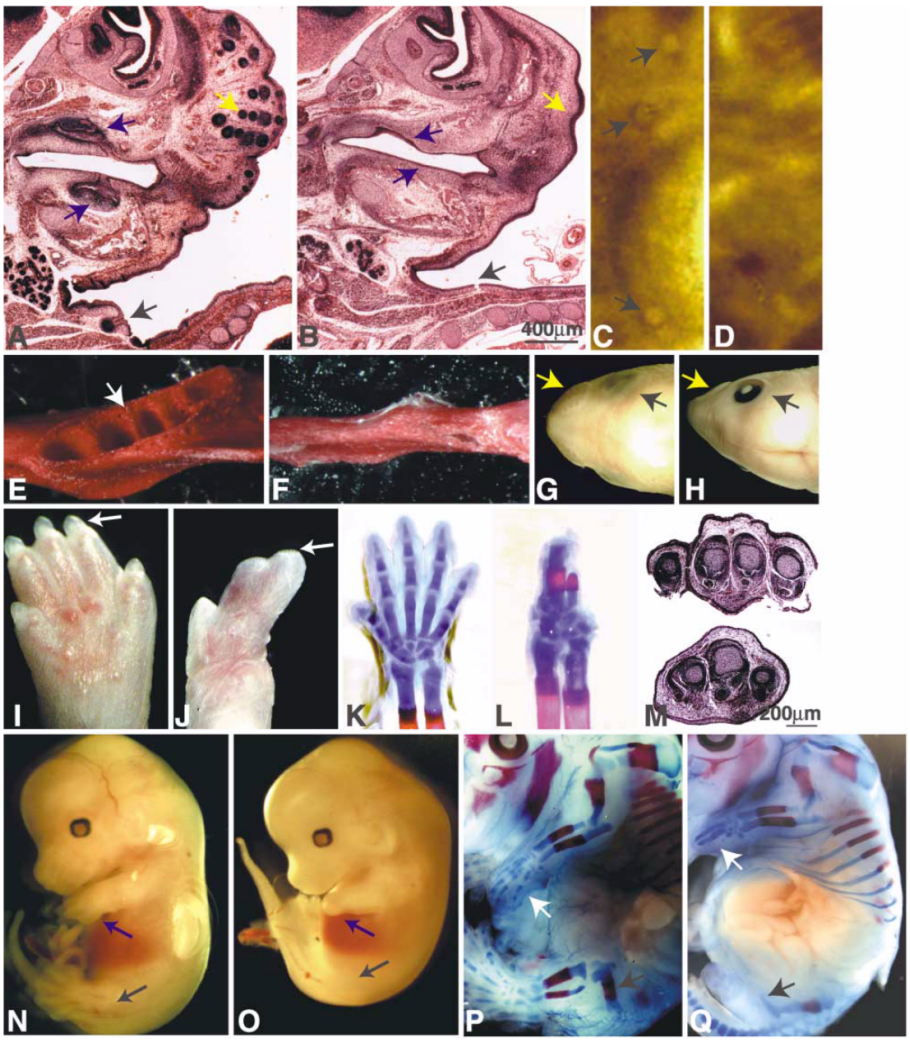

Figure 6. Pleiotropic Effects of the K14-*Dkk1* Transgene

(A and B) Sagittal sections through the head region of nontransgenic (A) and high-expressing K14-*Dkk1* transgenic (B) founder embryos at E14.5. Vibrissa follicles (yellow arrows) and mammary glands (black arrows) are absent in the transgenic embryos. The development of molar teeth has reached the cap stage in the nontransgenic embryos ([A], blue arrows). In the transgenic embryos, some thickening of the oral epithelium has occurred ([B], upper blue arrow), but tooth development is arrested before the bud stage.

(C and D) Ventral skin dissected from nontransgenic (C) and high-expressing transgenic founder (D) embryos at E13.5 and viewed under a light microscope. Mammary buds are visible in nontransgenic skin (arrows) but are absent from the same region of transgenic skin.

(E and F) Alveolar ridges present in the mandible of a nontransgenic mouse from line #7 at P28 ([E], white arrow) are absent from the mandible of a transgenic littermate (F).

(G and H) Nontransgenic (G) and high-expressing transgenic founder (H) embryos at E18.5. The transgenic embryo has open eyes (black arrows) and absence of the vibrissa pad (yellow arrows).

(I and J) Appearance of the distal forelimb of a newborn high-expressing transgenic founder (J) showing missing digits and absence of nails (white arrow) compared with the forelimb of a nontransgenic littermate (I).

(K and L) Skeletal preparations of the distal forelimb from a high-expressing transgenic founder embryo at E18.5 (L) showing absent and fused digits, compared with the forelimb of a nontransgenic littermate (K).

(M) Transverse sections through the distal left forelimbs of nontransgenic (upper section) and high-expressing K14-*Dkk1* transgenic founder (lower section) embryos at E16.5. The transgenic limb has fewer digits.

(N and O) High-expressing transgenic founder embryo at E13.5 (O) showing abnormal forelimb (blue arrow) and failure of hindlimb outgrowth (black arrow) compared with a nontransgenic littermate (N).

(P and Q) Skeletal preparations of a high-expressing transgenic founder embryo at E16.5 (Q) showing reduced numbers of forelimb digits (white arrow) and truncation of the hindlimb at the midfemur (black arrow) compared with a nontransgenic littermate (P).

however, this may have been secondary to defective patterning of the distal limb, as mice expressing high and hindlimbs (Figures 6I–6M and data not shown). In the most affected embryos, growth of the hindlimb bud

are similar to those described for chick limbs exposed to ectopic *Dkk1*, and are consistent with failure of maintenance of the apical ectodermal ridge (Mukhopadhyay et al., 2001).

Discussion

WNT signals have been implicated in the control of hair follicle development, but their precise roles and the extent to which they are required in different follicle types have been unclear. We show here that ectopic expression of *Dkk1* prevents the initiation of development of vibrissae and all types of hair follicles. Since DKK1 is a specific inhibitor of WNT coreceptors of the LRP family that are required for activation of the canonical WNT signaling pathway (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001; Zorn, 2001), our results indicate that canonical WNT signals are universally required for initiation of follicle development.

Current analyses of hair follicle development suggest that the establishment of a regular array of placodes in the surface epithelium in response to the first dermal message is achieved through the competing activities of molecules that promote or repress placode fate (reviewed in Millar, 2002). Our finding that ectopic expression of *Dkk1* blocks localized expression of known placode markers indicates that canonical WNT signals are required for this process. These WNT signals are necessary for localized expression of *Edar* and *Lef1*. *Wnt10b* lies genetically downstream of EDA/EDAR signaling in primary hair follicle placodes (Figure 5) but not vibrissa follicles, in which expression of *Wnt10b* may be regulated by genes related to *Eda* and *Edar* (Kojima et al., 2000; Yan et al., 2000), or by an entirely different signaling pathway. Activation of WNT signaling in the epithelium controls localized accumulation of *Shh*, which regulates follicle development, and *Bmp*s, which repress follicle fate (Botchkarev et al., 1999; Chiang et al., 1999; Huelsken et al., 2001; Jiang et al., 1999; Jung et al., 1998; Noramly and Morgan, 1998; St-Jacques et al., 1998). Expression of *Dkk1* can be induced by BMP signaling (Mukhopadhyay et al., 2001), suggesting that endogenous expression of *Dkk1* in mesenchymal cells surrounding developing follicles may be regulated by *Bmp*s expressed in the placode and dermal condensate, and might mediate some of the placode-repressing functions of *Bmp*s.

Two models may explain the effects of ectopic *Dkk1* in developing skin, based on inhibition of WNT signaling in the dermis or epidermis. In the first model, activation of the canonical WNT signaling pathway in dermal cells is required for generation of the first dermal message. In the second model, activation of the canonical WNT pathway in the epidermis is required for the initial response of epithelial cells to the first dermal message.

The first model is consistent with the observation that nuclear β-catenin appears in the dermis of the chick feather tract 1 day before it appears in the epithelium (Noramly et al., 1999), and with the finding that *Lef1* is required in the mesenchyme for the initiation of vibrissa 1996). The feather-inducing capabilities of dorsal dermis in the chick are conferred by signals from the dorsal neural tube that can be substituted by WNT1, suggesting that WNT signals from the neural tube are necessary for dorsal dermis to become inductive (Olivera-Martinez et al., 2001); possible sources of WNTs that might regulate the inductive capacities of the dermis in other body regions have yet to be identified. According to this model, diffusion of DKK1 from the epithelium to the dermis in K14-*Dkk1* transgenic mice would block generation of the first dermal message. In mice bearing a K14-Cre-mediated deletion of the cell-autonomous β-*catenin* gene (Huelsken et al., 2001), WNT signaling is affected only in the epithelium, providing a possible explanation for the later block to hair follicle development observed in these mice.

The second model, in which the WNT signaling pathway is activated in the epithelium as an initial response to the first dermal message, is not consistent with the conclusions of Huelsken et al. that WNT signaling in the epithelium lies downstream of EDA/EDAR signaling (Huelsken et al., 2001). These authors detected localized expression of *Edar* and *Lef1* in the epidermis at E15.5 in the absence of β-catenin expression in mice bearing a K14-Cre-mediated knockout of β-*catenin*. However, in these experiments, deletion of β-*catenin* was inefficient and progressive in embryonic epidermis, leaving open the possibility that *Edar* and *Lef1* expression was initiated prior to deletion of the β-*catenin* gene and depletion of β-catenin protein. Thus, it remains possible that early activation of the canonical WNT signaling pathway in the epithelium by *Wnt*s expressed in the epithelium or upper dermis regulates initiation of patterned *Edar* and *Lef1* expression. Although the two *Wnt* genes expressed in embryonic dermis, *Wnt11* and *Wnt5a* (Reddy et al., 2001), do not activate canonical signaling in most assays (Niehrs, 2001), several canonical *Wnt*s, including *Wnt10b*, are uniformly expressed in the epithelium prior to placode formation (Reddy et al., 2001) and might be candidates for these signals. In this scenario, the observation that K14-*Dkk1* expression blocks hair follicle development at an earlier stage than K14-Cre-mediated deletion of β-*catenin* could be explained by the high and uniform levels of *Dkk1* expression in transgenic K14-*Dkk1* skin at E14.5 (Figure 4B), and by the fact that the inhibitory effects of DKK1 protein are likely to be immediate. Our data are therefore consistent with inhibition by DKK1 of either generation of, or the initial response to, the first dermal message, and further experiments will be required to distinguish between these mechanisms.

Despite the dramatic effects of *Dkk1* expression on hair follicle formation, obvious abnormalities of the epidermis and dermis were not observed in K14-*Dkk1* transgenic mice. Thus, although several *Wnt* genes are expressed in embryonic interfollicular skin (Reddy et al., 2001), our results suggest that canonical WNT signaling does not regulate epidermal differentiation. Since the effects of *Dkk1* are specifically to suppress the canonical WNT pathway (Semenov et al., 2001; Wehrli et al., 2000; Zorn, 2001), noncanonical signaling activated by some of these WNTs may play roles in the skin that would not have been revealed in our studies. Since mice uniformly expressing high levels of *Dkk1* in the skin die soon after

long-term consequences of global loss of canonical WNT signaling on epidermal function, including possible effects on the maintenance of epidermal stem cells (Zhu and Watt, 1999).

In addition to preventing initiation of hair follicle development, ectopic *Dkk1* blocks the formation of teeth and mammary glands which, like hair follicles, develop through inductive interactions between the surface epithelium and mesenchyme. The first morphological sign of tooth formation is thickening of the oral epithelium to form a dental lamina, which subsequently buds into the underlying mesenchyme and then undergoes folding morphogenesis during the cap and bell stages (reviewed in Jernvall and Thesleff, 2000). *Lef1* is required in the epithelium for the transition from bud to cap stage (Kratochwil et al., 1996), suggesting a role for WNT signaling in regulating formation of the cap stage tooth. In K14-*Dkk1* transgenic embryos tooth buds fail to develop, indicating that canonical WNT signals are also required for the transition from dental lamina to bud stage. *Wnt*s *-4*, *-6*, and *-10b* are expressed in presumptive dental epithelium (Sarkar and Sharpe, 1999), and are possible candidates for these signals.

Mammary buds are thought to form by migration of epithelial cells along a line running from anterior to posterior between the limb buds (Propper, 1978). *Lef1* null mice develop a reduced number of mammary buds, and development of those that do form arrests at the bud stage (van Genderen et al., 1994). These results, together with our finding that mammary buds fail to form in the presence of ectopic *Dkk1*, indicate that canonical WNT signals are required for induction of mammary gland development. Consistent with this hypothesis, *Wnt10b* is expressed in the mammary line, with expression becoming restricted to mammary buds as they develop (Christiansen et al., 1995).

The limb, hair, tooth, and mammary gland phenotypes of K14-*Dkk1* mice are more severe than those described for *Lef1* null mice, which lack limb defects and in which development of some hair follicles and mammary buds is initiated, and teeth develop to the bud stage (van Genderen et al., 1994). Redundancy of LEF1 with TCF1 in limb development has been previously described (Galceran et al., 1999); our data suggest that LEF1 is also at least partially redundant with other LEF/TCF family member(s) in the initial stages of hair follicle, tooth, and mammary gland development.

Previous studies have addressed the functions of the downstream WNT effector molecules LEF1 and epithelial β-catenin in appendage development (Huelsken et al., 2001; Kratochwil et al., 1996; van Genderen et al., 1994). Here, by using a secreted inhibitor that acts at the level of essential WNT coreceptors, we show that signaling by canonical WNT proteins is required for the initiation of formation of all types of hair follicles, for formation of mammary buds, and for development of teeth beyond the dental lamina stage. Our results demonstrate that the actions of canonical WNT proteins in the skin precede localized expression of genes that regulate hair follicle placode formation, and indicate that canonical WNT signaling is required for the generation

Experimental Procedures

Generation of K14-*Dkk1* Transgenic Mice

The mouse *Dkk1* coding region was amplified by PCR of E14.5 cDNA using the following primers, containing BamHI sites for subcloning: 5′-CCCGGATCCGCGTCCTTCGGAGATGATGG-3′ and 5′-AATGGATCCTTTAGACTGTCGGTTTAGTGTCTC-3′. The *Dkk1* cDNA was cloned into a K14 expression vector identical to that described in Saitou et al., 1995. The transgene was released by digestion with EcoRI and HindIII and microinjected into fertilized eggs from a B6SJLF1/J x B6SJLF1/J cross (Jackson Laboratories). Transgenic mice or embryos were identified by Southern blotting of tail biopsy or yolk sac DNA using a *Dkk1* cDNA probe. Founder mice were bred to nontransgenic littermates or to FVB/N mice (Jackson Laboratories).

Histology, In Situ Hybridization, BrdU Labeling, and Immunofluorescence

Tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 5 μm for hematoxylin and eosin staining, in situ hybridization, or immunofluorescence (Millar et al., 1999). *Wnt10b*, *Shh*, and *Gli1* in situ hybridization probes were synthesized from cDNA clones (Wang and Shackleford, 1996; St-Jacques et al., 1998). Antisense and sense probe templates for *Dkk1*, β-*catenin*, *Lef1*, *Wnt6*, *Fz6*, *Bmp2*, *Bmp4*, *Bmp7*, *Edar*, and *K17* were synthesized by PCR of E14.5 mouse cDNA, using primers containing T7 RNA polymerase binding sites to amplify the following sequences: *Dkk1*: NM_010051, nt 24–869; β-*catenin*: M90364, nt 2301–2619; *Lef1*: X58636, nt 1921–2380; *Wnt6*: M89800, nt 1–257; *Fz6*: U43319, nt 1863–2271; *Bmp2*: NM_007553, nt 1141–1535; *Bmp4*: X56848, nt 1011–1573; *Bmp7*: NM_007557, nt 1144–1485; *Edar*: AF160502, nt 302–1038; *K17*: AB013608, nt 1263–1505. For BrdU labeling, mice were injected with 50 μg BrdU/g body weight and sacrificed after 1 hr. Skin sections were dewaxed, microwaved in 10 mM sodium citrate, and incubated with anti-BrdU antibody (Roche), followed by incubation with biotinylated goat anti-mouse antibody and Texas red-conjugated avidin (Vector Laboratories). Three control and three transgenic samples were assayed for each time point; for each, the number of positive cells in the basal layer of the epidermis was counted in each of ten fields at 10× magnification, and the average number per field was calculated. For immunofluorescence, sections were incubated with rabbit polyclonal antibodies against K1, K10, K6, involucrin, loricrin, filaggrin (all from Covance), or K17 (McGowan and Coulombe, 1998), followed by detection with biotinylated goat anti-rabbit IgG and Texas red-conjugated streptavidin (Vector Laboratories). Sections were counterstained with 2 μg/ml Hoechst 33258 (Sigma). In situ hybridization experiments were photographed using an MVI Darklite stage adaptor.

Analysis of $Eda^{Ta\text{-}6J}$ Mutant Embryos

Female C57BL/6J-$A^{w\text{-}J}$-$Eda^{Ta\text{-}6J/+}$ mice (Jackson Laboratories) were mated to FVB/N males and sacrificed 14 or 15 days after appearance of a copulation plug (day 0.5). Embryos were sexed by PCR genotyping of tail biopsy DNA using the following primers for the Y-specific gene *Zfy* (Ashworth et al., 1989): 5′-AAGATAAGCTTACATAATCACATGGA-3′ and 5′-CCATATGAAATCCTTTGCTGCACATGT-3′. Male embryos were genotyped for *Eda* by amplifying nt 2661–2980 from exon 1 of the *Eda* gene (GenBank accession number Y13438) and sequencing the PCR products. Mutant embryos were identified by deletion of a thymidine residue at nucleotide 2924 (GenBank accession number Y13438; 550delT in Srivastava et al., 1997). Mutant and wild-type littermates were processed for in situ hybridization with the *Wnt10b* probe as described above.

Skeletal Preparations

Embryos or limbs were fixed in 95% ethanol, stained with Alcian blue for cartilage, macerated in 1% potassium hydroxide, stained with Alizarin red for bone, and cleared in graded solutions of potassium hydroxide in glycerin.

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