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DEVELOPMENT AND DISEASE

Regulation of hair follicle development by the TNF signal ectodysplasin and its receptor Edar

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SUMMARY

X-linked and autosomal forms of anhidrotic ectodermal dysplasia syndromes (HED) are characterized by deficient development of several ectodermal organs, including hair, teeth and exocrine glands. The recent cloning of the genes that underlie these syndromes, ectodysplasin (*ED1*) and the ectodysplasin A receptor (*EDAR*), and their identification as a novel TNF ligand-receptor pair suggested a role for TNF signaling in embryonic morphogenesis. In the mouse, the genes of the spontaneous mutations *Tabby* (*Ta*) and *downless* (*dl*) were identified as homologs of *ED1* and *EDAR*, respectively. To gain insight into the function of this signaling pathway in development of skin and hair follicles, we analyzed the expression and regulation of *Eda* and *Edar* in wild type as well as *Tabby* and *Lef1* mutant mouse embryos. We show that *Eda* and *Edar* expression is confined to the ectoderm and occurs in a pattern that suggests a role of ectodysplasin/Edar signaling in the interactions between the ectodermal compartments and the formation and function of hair placodes. By using skin explant cultures, we further show that this signaling pathway is intimately associated with interactions between the epithelial and mesenchymal tissues. We also find that *Ta* mutants lack completely the placodes of the first developing tylotrich hairs, and that they do not show patterned expression of placodal genes, including *Bmp4*, *Lef1*, *Shh*, *Ptch* and *Edar*, and the genes for β-catenin and activin A. Finally, we identified activin as a mesenchymal signal that stimulates *Edar* expression and WNT as a signal that induces *Eda* expression, suggesting a hierarchy of distinct signaling pathways in the development of skin and hair follicles. In conclusion, we suggest that *Eda* and *Edar* are associated with the onset of ectodermal patterning and that ectodysplasin/edar signaling also regulates the morphogenesis of hair follicles.

Key words: Ectodermal dysplasia, *Tabby*, *downless*, Ectodysplasin, *Eda*, Edar, TNF, LEF1, Activin, BMP, SHH, WNT, Hair follicle, EGF, *ED1*, FGF, Mouse

INTRODUCTION

Reciprocal interactions between the epithelial and mesenchymal tissues constitute a central mechanism, which determines the locations, sizes and shapes of organs. Hair follicle morphogenesis is initiated by signals from the embryonic dermis, which instructs the overlying ectoderm to initiate placode formation (Hardy, 1992). The placodes send messages back to the underlying mesenchyme, which forms dermal condensates that in turn signal back to the epithelial keratinocytes regulating the morphogenesis of the follicle. The epithelial-mesenchymal interactions that regulate the formation of hair follicles are mediated by the same conserved signal molecules as in other epithelial appendages. These signals belong mostly to four conserved families: transforming growth factor β family (TGFβ), fibroblast growth factors (FGFs), hedgehog family (HH) and WNT family (Gurdon, 1992; Gat et al., 1998; Jernvall and Thesleff, 2000; Kishimoto et al., 2000). In addition, the Notch pathway has been implicated in lateral signaling within the ectodermal and mesenchymal tissue compartments (Chen et al., 1997). It is noteworthy that signals in the different families are used reiteratively during organogenesis (Gat et al., 1998; Noramly et al., 1999; Jernvall and Thesleff, 2000). Furthermore, most of the same signaling molecules are also expressed in the adult follicles and are implicated in the mediation of the hair cycle.

Tumor necrosis factors (TNF) have been recently added to the list of growth factors regulating hair follicle morphogenesis. TNFs have been traditionally associated with host defense, immunity, inflammation and cancer (Gruss and Dower, 1995), and more recently with the regulation of osteoclast differentiation (Filvaroff and Derynck, 1998) and endothelial survival/vascular homeostasis (Malyankar et al., 2000). Their function in embryonic organogenesis was

revealed by the cloning of genes behind ectodermal dysplasia syndromes in humans and mice. Ectodermal dysplasias comprise over 100 syndromes, which are characterized by defective development of ectodermal organs, including hair, teeth and exocrine glands. The anhidrotic ectodermal dysplasia (HED) is the most common among these syndromes. The positional cloning of the gene behind X-linked HED (Kere et al., 1996), and subsequent cloning of the gene responsible for its mouse counterpart *Tabby* (*Ta*; the gene is called *Eda* – Mouse Genome Informatics) (Ferguson et al., 1997; Srivastava et al., 1997) led to the identification of a novel TNF ligand, ectodysplasin (Mikkola et al., 1999). The gene behind another spontaneous mouse mutant, *downless* (*dl*), which shares a similar phenotype to *Ta*, encodes a new member of the TNF receptor (TNFR) family, called Edar (Headon and Overbeek, 1999). Edar was shown to be mutated in individuals with autosomal dominant and recessive forms of HED (Monreal et al., 1999), and it was subsequently demonstrated to be the receptor for ectodysplasin (Tucker et al., 2000; Kumar et al., 2001).

The spontaneous mouse mutants *Ta*, *dl* and *crinkled* (the gene responsible encodes the death domain adapter, Edaradd) (Headon et al., 2001) have similar phenotypes described in detail already in the 1950s and 1960s (Falconer et al., 1951; Grüneberg, 1965; Sofaer, 1969). They have missing and abnormally shaped teeth and lack several exocrine glands (Grüneberg, 1971). Their hair development is generally delayed, tail hairs are partially or completely absent, some whiskers are lacking and they completely lack certain types of hair (Grüneberg, 1966). Wild-type mouse coat has four types of hair: tylotrichs, awls, zigzags and auchenes, whereas *Ta* mutants have only hairs resembling abnormal awls in their pelage (Grüneberg, 1966; Kindred, 1967; Claxton, 1967; Hardy, 1969). The tylotrich (guard) hairs that are missing in *Ta* mutant skin form as the first wave of hair follicles and their initiation is seen in wild-type mice at E14 (Mann, 1962). It has been shown previously that, *Ta* skin shows no signs of follicle initiation at this stage, and the first hair placodes resembling stage 1 and 2 awl placodes can be seen in E16 *Ta* embryos (Vielkind and Hardy, 1996).

All organs affected in individuals with HED, and in *Ta* and *dl* mutant mice, develop as ectodermal appendages, and the molecular mechanisms regulating their morphogenesis are shared to a great extent (Thesleff et al., 1995). We have previously analyzed the expression and regulation of *Eda* and *Edar* during tooth morphogenesis, as well as the pathogenesis of the tooth phenotype of *Ta* mice (Pispa et al., 1999; Laurikkala et al., 2001). These studies indicated that Eda/Edar signaling regulates the function of the epithelial signaling centers during tooth morphogenesis and is associated with epithelial-mesenchymal interactions (Laurikkala et al., 2001). The aim of this study was to analyze the functions of this signaling during skin and hair follicle development. The expression patterns of *Eda* and *Edar* during hair follicle morphogenesis indicate that, as in teeth, their signaling mediates cell interactions mainly within the epithelial cell layer rather than across epithelial-mesenchymal boundaries. The analysis of cultured embryonic skin indicated that both *Eda* and *Edar* expression required the presence of mesenchymal tissue. The analysis of local application of several signaling molecules on the explants showed that activin A stimulated *Edar* expression, but only when mesenchymal tissue was present. *Eda* expression in the epithelium was induced by WNT signaling even in the absence of mesenchymal tissue. The WNT signals were shown to be transduced by LEF1, because *Eda* expression was absent from the epidermis of *Lef1*$^{-/-}$ embryos. We conclude that ectodermal TNF signaling, which is mediated by Eda and Edar, regulates the formation and/or function of hair placodes and is integrated with WNT and activin signal pathways linking TNF functions to epithelial-mesenchymal interactions.

MATERIALS AND METHODS

Animals and collection of tissues

The wild-type mice were NMRI and *Ta* mice were B6CBACa-A$^{w\text{-}J}$/A-Ta (stock number JR 0314; Jackson Laboratories, Bar Harbor, ME). Their maintenance has been described previously (Pispa et al., 1999). The generation and analysis of the *Lef1*$^{-/-}$ mice have been described previously (van Genderen et al., 1994; Kratochwil et al., 1996). Staged embryos from embryonic day 11 (E11) to newborn (NB) were collected. The tissues were fixed overnight in 4% paraformaldehyde (PFA) and taken through ethanol series and xylene into paraffin and serially sectioned at 7 μm for histology, immunohistochemistry and in situ hybridization. Sections for normal histology were stained with Hematoxylin and Eosin.

Organ cultures

Back skin was dissected from E13, E14 and E15 wild-type mouse embryos in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) under a stereomicroscope. Skin explants were cultured either as whole explants or the epithelium was separated from the mesenchyme after incubation in 2.25% trypsin/0.75% pancreatin (15-20 minutes; 37°C; Gibco). The explants were grown for 24 hours on Nuclepore filters at 37°C in a Trowell type culture containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS, PAA Laboratories GmbH) and 1% penicillin-streptomycin (Gibco, Paisley, Scotland).

Recombinant proteins and bead implantation assays

Heparin acrylic beads (Sigma, St Louis, MO) were used with FGFs and Affi-Gel agarose beads (BioRad) with other signal molecules. About 100 beads were washed with PBS and soaked in 10 μl of growth factor for 45 minutes at 37°C. The recombinant proteins used for gene expression induction studies were activin A (50 ng/μl; R&D Systems, Abingdon, UK), BMP4 (75 ng/μl; a kind gift from J. Wozney, Genetics Institute, Cambridge, MA), EGF (25 ng/μl; Boehringer Mannheim GmbH, Germany), FGF10 (25 ng/μl; R&D), SHH (50 ng/μl; R&D) or bovine serum albumin (BSA, 1 μg/μl; Sigma). The NIH3T3 cell line expressing *Wnt6* gene was a kind gift from Seppo Vainio (University of Oulu, Finland); the generation of this cell line has been described previously (Kettunen et al., 2000). The beads or cell aggregates were placed on top of or under the explants using fine forceps, and the explants were cultured and fixed as described earlier (Kettunen et al., 2000). Tissues were treated with 100% methanol for 2 minutes, fixed in 4% PFA (overnight; 4°C) and processed further.

In situ hybridization

Radioactive in situ hybridization for paraffin wax-embedded sections was performed as described earlier (Wilkinson and Green, 1990). Probes were labeled with ^{35}S-UTP (Amersham); exposure time was 12 days. Whole mount in situ hybridization was as described earlier (Raatikainen-Ahokas et al., 2000). Color reaction was detected by using BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim Gmbh, Germany).

The probes used detected murine activin βA (a kind gift from Olli Ritvos) (Erämaa et al., 1992); a 414 bp fragment of murine β-catenin mRNA (nucleotides 135-549) [amplified using primers 5′CATGGAGCCGGACAGAAAAGCTGCTG3′ and 5′CGTGTGG-AAGTTCCGCGTCATCCTG3′, and cloned into pCRII-TOPO (Invitrogen)]; murine *Bmp4* (Vainio et al., 1993); murine *Edar* (Laurikkala et al., 2001); murine *Lef1* (Travis et al., 1991); murine *Ptch* (Kim et al., 1998); rat *Shh* (Vaahtokari et al., 1996); murine *Wnt6* (Weber-Hall et al., 1994); murine *Wnt10a* (Dassule and McMahon, 1998); and murine *Eda* (Laurikkala et al., 2001).

Immunostaining

Immunoreactivity of antigens in 4% PFA fixed sections was restored by autoclaving slides in 10mM sodium citrate, pH 6 (120°C; 30 minutes). To block endogenous peroxidase activity of skin, the sections were treated with 0.3% H_2O_2 in methanol and rinsed with PBS. Primary antibodies against the following proteins were used: polyclonal antibody against keratin 14 (1:400) (a kind gift from Elaine Fuchs), keratin 10 (1:400) and filaggrin (1:200) (both from Berkeley Antibody Company, BAbCo). Sections were incubated with primary antibodies diluted in PBS containing 0.1% BSA, 1% normal goat serum and 0.1% Triton-X (overnight; 4°C). Subsequent immunostaining procedures were performed using Vectastain Elite ABC kit (Vector Laboratories). Color development was detected by using AEC staining method.

RESULTS

Expression of *Eda* and *Edar* during hair follicle development

Eda and *Edar* expression have been previously localized in the ectoderm and hair follicles of developing mouse skin (Srivastava et al., 1997; Headon and Overbeek, 1999), but detailed expression patterns and comparisons of the two genes have not been reported. We analyzed *Eda* and *Edar* expression by in situ hybridization in tissue sections and whole mounts of back skin of wild-type mice from E11 to birth. Both *Eda* and *Edar* transcripts were detected throughout the simple ectoderm layer covering the E11-E13 embryos (Fig. 1A,B, see Fig. 5I and results not shown). At E14, *Edar* expression became punctuated, and at E15, when placodes had formed as localized thickenings of epithelium, *Edar* expression was strongly upregulated in the basal epithelial cells in these foci, whereas it was downregulated in the rest of the ectoderm (Fig. 1D,F, see Fig. 7A). These first placodal thickenings give rise to the tylotrich hair follicles (Mann, 1962). At this stage the placodes were devoid of *Eda* transcripts, but *Eda* expression continued in the rest of the epidermis (Fig. 1E). At E17, *Edar* expression was intense in the bulbs of the maturing stage 3-4 hair follicles of tylotrich hairs and *Eda* was also expressed in this tissue showing partial co-expression with *Edar* (see Fig. 5E,K). *Edar* was also expressed in the initiated epithelial placodes of the awl hairs (not shown). *Eda* transcripts were detected in the interfollicular epidermis (not shown). *Eda* and *Edar* transcripts colocalized in the maturing follicles of newborn (P0) mouse (Fig. 1G,H). Although *Eda* expression appeared to be confined to the epithelium of embryonic skin, we cannot exclude the presence of some *Eda* transcripts in the mesenchyme. We conclude that the TNF signaling mediated by Eda and by Edar takes place mainly within the epithelial tissue rather than across epithelial-mesenchymal tissue layers. Of special interest is the co-expression of *Edar* with several signal molecules in the placodes of hair follicles (see Fig. 7), which suggests that ectodysplasin from the interfollicular ectoderm may regulate the functions of the signaling centers at the placodes.

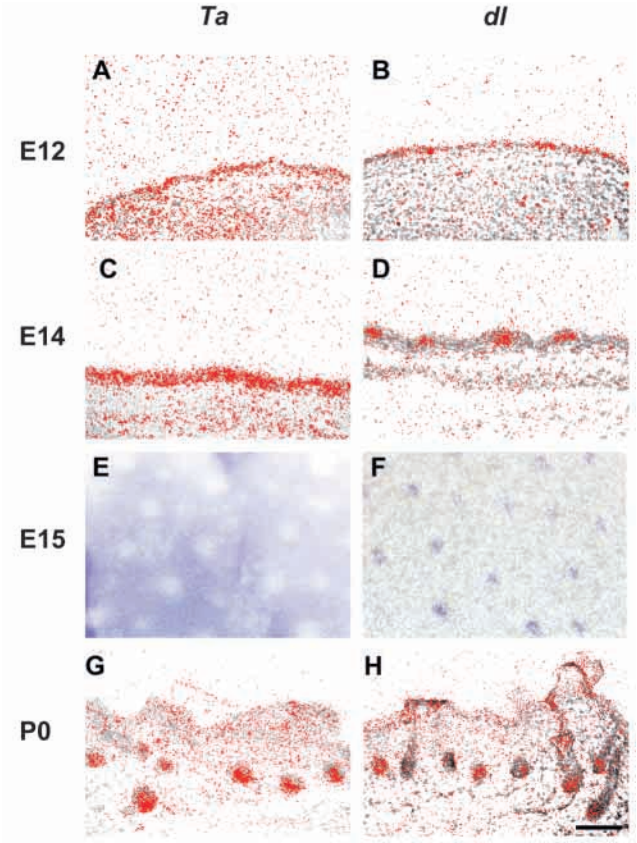

Fig. 1. Expression of *Eda* (*Ta*, encoding the TNF ectodysplasin) and *Edar* (*dl*, encoding the TNF receptor Edar) during hair follicle development in the back skin. (A,B) *Eda* and *Edar* transcripts are co-expressed in the E12 embryonic ectoderm. (C-F) At E14-15, *Eda* expression continues in the ectoderm except in the newly formed placodes. The placodes express *Edar* intensely, whereas it is downregulated in the rest of the ectoderm. (G-H) *Eda* and *Edar* transcripts are intensely expressed in the bulb of newborn stage 3-6 hair follicles. *Eda* expression continues in the epidermis. Scale bar: 100 μm.

Regulation of *Eda* and *Edar* expression by mesenchyme and signal molecules

We studied the role of epithelial-mesenchymal interactions in the regulation of *Eda* and *Edar* expression in explant cultures of E13, E14 and E15 back skin, which represent the stages when placode formation starts. Whole skin explants were cultured, or epithelium and mesenchyme were cultured in isolation. The expression of *Eda* and *Edar* was analyzed after 24 hours of culture by whole-mount and sectional in situ hybridization. When the ectoderm was cultured alone both *Eda* and *Edar* expression was downregulated (not shown). However, when the whole skin explant was cultured, the expression of *Eda* and *Edar* was evident in radioactive in situ hybridization of sections, but in whole mounts it was faint (Fig. 2, see Fig. 4, and data not shown).

We then analyzed the effects of several potential signal molecules on *Eda* and *Edar* expression. These were activin A

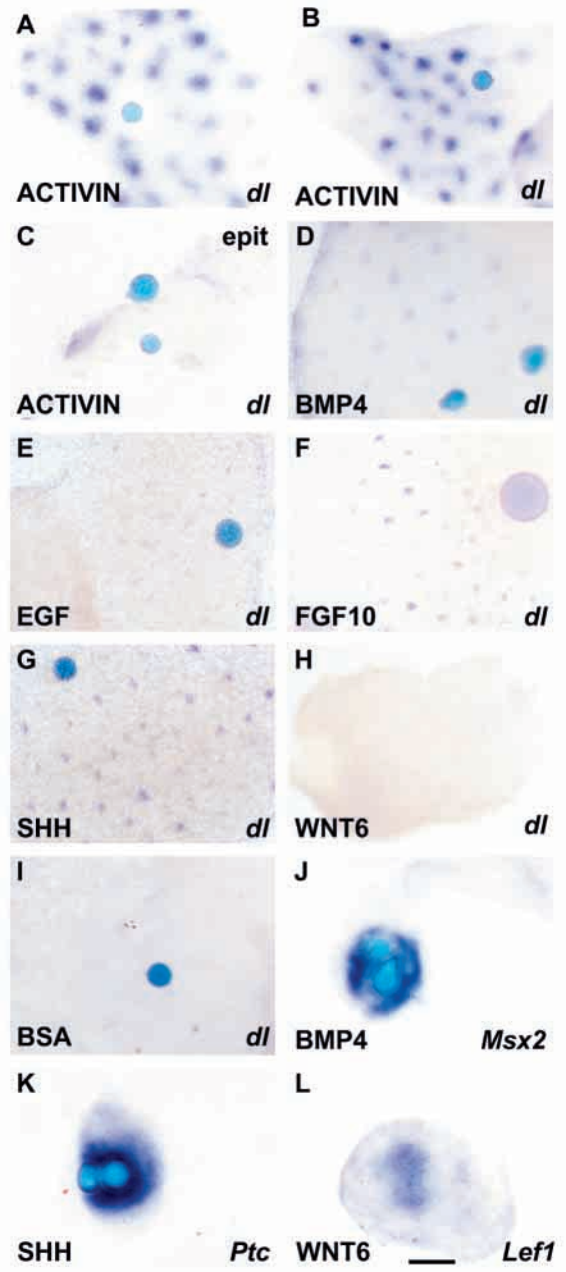

Fig. 2. Stimulation of *Edar* expression (*dl*) in hair placodes by activin A but not by other signals. (A,B) *Edar* expression is stimulated by an activin A-releasing bead in placodes throughout the whole skin explant. (C) Activin A does not induce *Edar* expression in isolated skin epithelium. (D-I) None of the other tested signal proteins [BMP4, EGF, FGF10, SHH and WNT6 (introduced by cell aggregates)] or BSA control beads stimulated *Edar* expression. (J-L) Positive controls: (J) *Msx2* expression induced by BMP4. (K) *Ptch* (*Ptc*) induced by SHH. (L) *Lef1* induced by WNT6-expressing cells. Scale bar: 200 μm.

and BMP4 (TGFβ family), SHH, FGF10, WNT6 and EGF. Activin A mutant mice lack whiskers (Matzuk et al., 1995). SHH, as well as members of the BMP, FGF and WNT families, have been previously associated with hair and feather morphogenesis (Oro and Scott, 1998; Noramly et al., 1999; Kishimoto et al., 2000). A link between EGF and *Eda* has been

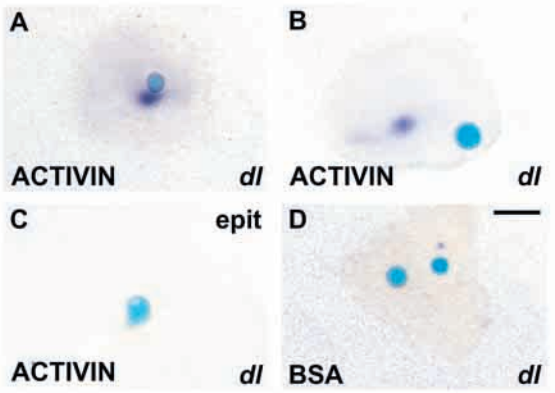

Fig. 3. Stimulation of *Edar* (*dl*) expression by activin A in dental epithelium. (A,B) Activin A bead has stimulated *Edar* expression in the epithelial signaling center, the enamel knot in an E12 whole tooth germ. (C) *Edar* expression is not induced by activin A in isolated tooth epithelium. (D) BSA control beads did not stimulate *Edar* expression. Scale bar: 200 μm.

previously proposed, because EGF injections can rescue the sweat gland phenotype in newborn *Tabby* mice (Blecher et al., 1990). We have previously shown that FGF10 partly rescues the tooth phenotype in *Tabby* mice (Pispa et al., 1999) and that activin A and WNT6 stimulate *Edar* and *Eda* expression, respectively, in the dental epithelium (Laurikkala et al., 2001).

WNT6 was applied by cell aggregates expressing the protein, whereas other signals were introduced by beads soaked in recombinant proteins. The cells and beads were placed either on top or under back skin explants of E13, E14 or E15 embryos. The explants included whole skin or isolated epithelium or mesenchyme.

Activin A stimulates *Edar* expression in the ectodermal placodes but requires the presence of mesenchyme

When the whole skin of E13, E14 and E15 embryos was cultured for 24 hours with the signal proteins, only activin A had an effect on *Edar* expression (Fig. 2A,B, and results not shown). It is noteworthy that expression was not induced around the bead, as in most bead induction assays (Fig. 2J,K) (Vainio et al., 1993). Instead, activin A caused an intense upregulation of *Edar* expression only in the placodes at apparently predetermined locations. However, activin did not stimulate *Edar* expression in isolated epithelium (Fig. 2C). The other signal molecules (BMP4, EGF, FGF10, SHH and WNT6) had no effects on *Edar* expression (Fig. 2D-H). BSA-soaked control beads (Fig. 2I) and 3T3 control cells (not shown) were also without effect.

This observation prompted us to re-examine our previous findings on the activin A-stimulated *Edar* expression in tooth germs. *Edar* is expressed in epithelial signaling centers also in teeth, but there is only one center per tooth germ, and we have shown previously by similar bead assays that activin A upregulated *Edar* in the dental epithelium of whole tooth explants (Laurikkala et al., 2001). A more careful analysis of several explants now indicates that, as in the skin explants, the site of stimulated *Edar* expression is predetermined and not directed by the bead. *Edar* expression was upregulated at different distances apparently correlating with the site of the

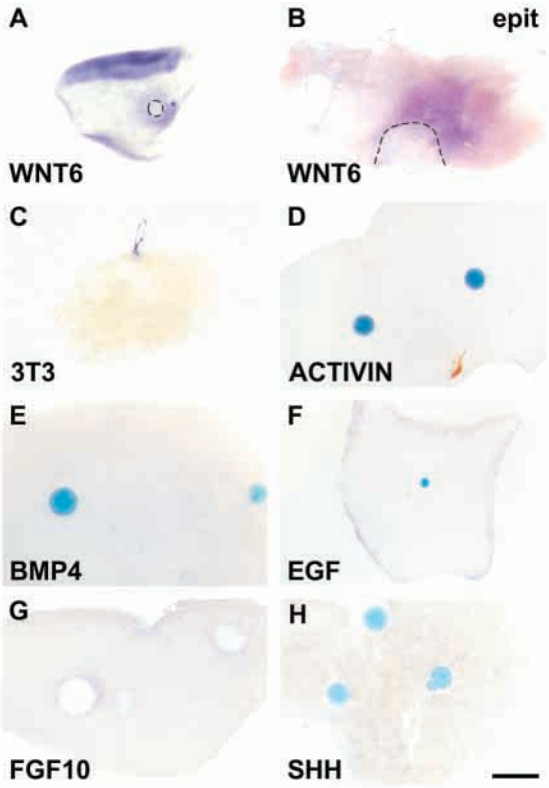

Fig. 4. Induction of *Eda* (*Ta*) expression by WNT6 in skin epithelium. (A,B) WNT6-expressing cells stimulate *Eda* expression in whole skin explants (A) as well as in isolated epithelium (B). (C) 3T3 control cells failed to induce *Eda*. (D-H) None of the other signals tested (activin A, BMP4, EGF, FGF10 and SHH) had stimulatory effects on *Eda* expression. Scale bar: 200 μm.

developing signaling center (Fig. 3A,B). Beads releasing activin A did not induce *Edar* expression in isolated tooth epithelium (Fig. 3C). BSA control beads produced no expression (Fig. 3D).

WNT6 induces *Eda* expression in the ectoderm

The potential of the various signaling molecules to regulate *Eda* expression was analyzed in a similar series of experiments, as described above for *Edar* expression. The cells expressing *Wnt6* stimulated the expression of *Eda* in the explants of whole back skin at all stages analyzed, as well as in isolated epithelium (Fig. 4A,B, and results not shown). The effect of *Wnt6*-expressing cells was similar when cultured on top of the explant and under it. None of the other signals had effects on *Eda* expression; BSA beads (not shown) and control 3T3 cells also had no effect (Fig. 4C-H; Table 1).

Eda expression requires LEF1

Canonical WNT signaling is mediated through the stabilization and accumulation of β-catenin and its consequent translocation to the nucleus, where β-catenin forms a complex with Tcf/LEF1 family of transcription factors (Eastman and Grosschedl, 1999; Bienz and Clevers, 2000). *Lef1*-deficient mice have defects in the development of several organs, among them hair, whiskers and teeth (van Genderen et al., 1994; Kratochwil et al., 1996). *Ta* mutant mice have defects in the same ectodermal organs, although the *Ta* phenotype is milder and there are differences in the types of hairs affected. Previously, we have shown that *Eda* expression was significantly, although not completely, downregulated in the tooth buds of *Lef1*$^{-/-}$ mice, whereas *Edar* expression was unaltered. As *Lef1* is expressed in the skin epithelium and mesenchyme at several stages of hair follicle development both in mesenchyme and epithelium, and colocalizes with both *Eda* and *Edar* expression in the early ectoderm and with *Edar* in the placodes (Fig. 1A-D,F,H, Fig. 7E,G) (Zhou et al., 1995), we investigated whether their expression is altered in the skin of *Lef1* knockout mice. No *Eda* expression was detected by in situ hybridization in the ectoderm at E11 and E13, while the wild-type littermates showed a clear signal (Fig. 5A,B, and results not shown). The analysis of *Lef1*$^{-/-}$ skin at E15, E17 and P0 indicated that *Eda* transcripts were mostly absent, although very faint expression was seen in the bulb of some follicles as well as in the ectoderm (Fig. 5D,F,H). *Edar* expression appeared normal in the early ectoderm, in the placodes as well as in the bulbs of hair follicles in *Lef1*$^{-/-}$ mice (Fig. 5J,L). These results are in line with our previous findings on tooth development (Laurikkala et al., 2001) and indicate that the WNT signals regulating *Eda* expression in the skin ectoderm are transduced by LEF1. However, our results indicate phenotypic differences between *Ta* and *Lef1* mutants, which appear to be in conflict with the requirement of LEF1 for *Eda* expression. *Edar* expression was patterned in *Lef1* mutants and histological analysis confirmed that the first tylotrich follicles, which are absent in *Ta* mutants, do develop in *Lef1*$^{-/-}$ embryos, although their number is reduced (Fig. 5F,H, and data not shown (van Genderen et al., 1994).

Table 1. Induction of *Eda* and *Edar* expression by signaling molecules in embryonic skin

	Induced gene				
Signaling molecule	*Eda*	*Edar*	*Lef1*	*Msx2*	*Ptch*
Activin A	0/9	11/12			
Epithelium alone	0/2	1/11			
Wnt6	12/12	0/12	8/8		
Epithelium alone	8/9	0/6			
BMP4	0/10	0/11		7/8	
EGF	0/8	0/7			
FGF10	0/6	0/9			
SHH	0/8	0/8			10/10
BSA	0/5	0/7			
3T3 cells	0/9	0/3			

Numbers indicate positive/total explants of E13, E14 and E15 back skin (no differences were detected in tissue responses at the different stages). *Lef1*, *Msx2* and *Ptch* were used as positive controls for Wnt6, BMP4 and SHH activity, respectively. Gene expression was analyzed by in situ hybridization after 24 hours in culture with beads releasing the signals or cells expressing the protein (Wnt6).

Development of hair follicles and differentiation of epidermis in *Ta* mutants

The phenotype and pathogenesis of skin and hair defects in *Ta* mice have been described earlier in detail (Sofaer, 1973; Hardy, 1969; Vielkind and Hardy, 1996). By plucking hairs, we confirmed that the hairs of *Ta* mutants look like awls whereas tylotrich (guard), auchene and zig zag hairs are absent (not shown). Our analysis of Hematoxylin and Eosin stained

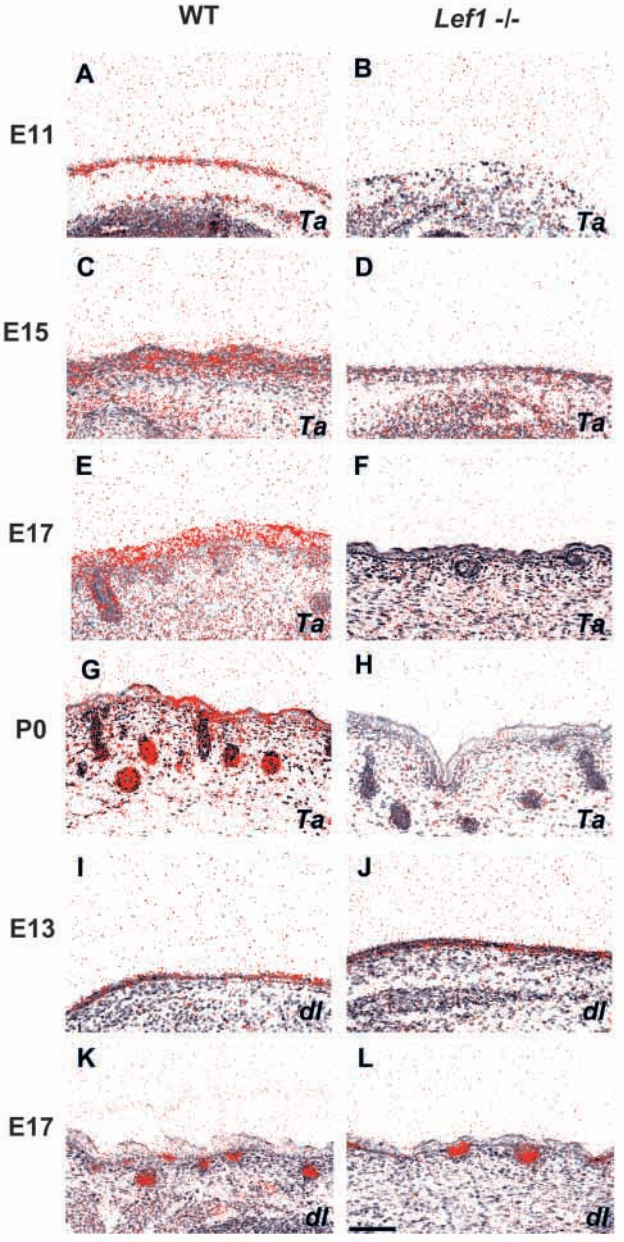

Fig. 5. Expression of *Eda* (*Ta*) and *Edar* (*dl*) in wild-type and *Lef1*$^{-/-}$ embryos. (A,C) *Eda* is expressed in the skin of E11 and E15 wild-type littermates of *Lef1*$^{-/-}$ embryos. (B,D) In the ectoderm of a *Lef1*$^{-/-}$ embryo, expression of *Eda* was almost completely absent. (E,G) *Eda* transcripts are seen in the epithelium as well as in the hair follicles in E17 and P0 wild-type littermates. (F) *Eda* transcripts are downregulated in *Lef1*$^{-/-}$ skin. (G) In P0, *Lef1*$^{-/-}$ skin, faint *Eda* expression is seen in the hair follicles. (I,J) *Edar* is expressed throughout the epithelium in E13 wild-type littermates and *Lef1*$^{-/-}$ embryos. (K,L) *Edar* transcripts are upregulated in the hair follicles in E17 wild-type littermates and *Lef1*$^{-/-}$ embryos. Scale bar: 100 μm.

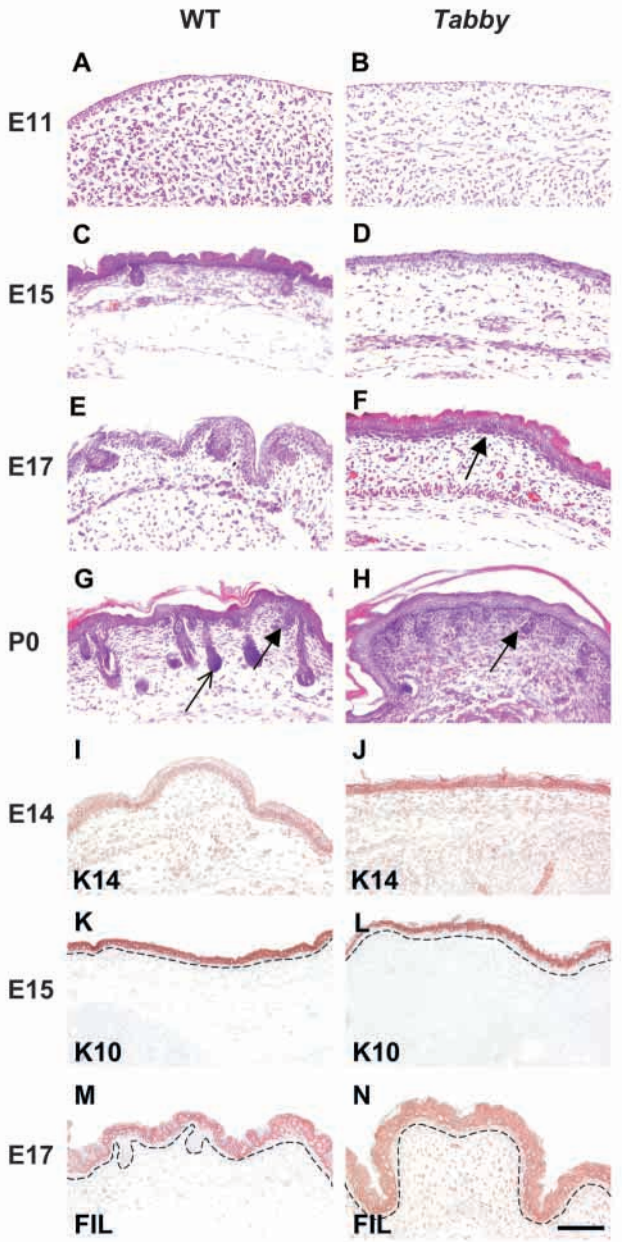

Fig. 6. Comparison of skin and hair follicle development between wild-type and *Ta* mutant embryos. (A,B) At E11, the ectoderm appears slightly thinner in *Ta* skin. (C,D) At E15, the first wave hair follicles (tylotrich or guard) are seen in wild-type skin whereas the *Ta* skin ectoderm is uniformly thin and devoid of hair placodes. (E,F) At E17, tylotrich hair follicles have developed into stage 3-4 in wild-type skin. Placodes of awl follicles (stage 1-2) have appeared in *Ta* skin (arrow) and the epidermis has increased in thickness. (G,H) At P0 (newborn), tylotrich (skeleton arrow) and awl follicles (arrow) are present in wild-type skin, whereas in *Ta* mice only awl follicles are seen. (I-N) Differentiation of the keratinocytes is not affected in *Ta* skin. (I,J) Keratin 14 is expressed in basal epithelial cells. (K,L) Keratin 10 is expressed in suprabasal cells. (M,N) Filaggrin (FIL) is expressed in differentiated keratinocytes. Broken lines indicate the interface between epithelium and mesenchyme. Scale bar: 100 μm.

sections confirmed the previously described phenotypic features of hair development in the embryos. At E11, the ectoderm layer appeared slightly thinner in the *Ta* embryo when compared with the wild type (Fig. 6A,B). The placodes of tylotrich hairs, which are seen as thickenings of ectoderm in wild-type mice at E14-E15, were not detected in *Ta* skin (Fig. 6C,D). The first signs of follicle formation were observed in *Ta* skin at E16.5-E17. These stage 1 and 2 follicles

presumably represent awl follicles, which appeared in the back skin of wild-type embryos at this age (Fig. 6E,F). In the wild-type embryos, the tylotrich follicles had developed into stages 3 and 4 (Fig. 6E). In newborn (P0) wild-type mice, the tylotrich follicles had reached stages 6 and 7 and the awl follicles stages 3-4. In addition, newly formed stage 1-2 hair follicles were present representing the zig zags and/or auchenes (Fig. 6G). Newborn *Ta* skin contained only stage 3 and 4 awl follicles (Fig. 6H).

It has been reported that the differentiation of the epidermal cell layers is accelerated in the *Ta* skin (Vielkind and Hardy, 1996). We analyzed the expression of epidermal differentiation markers in the *Ta* skin in order to examine whether their differentiation program was altered. The development of the ectodermal cell layer appeared rather delayed than advanced during early stages, and at E14 and E15 the superficial keratinizing layer was almost missing in *Ta* skin (Fig. 6C,D,I,J). However, epidermal development in *Ta* mice seemed to catch up, as no difference was observed later between *Ta* and wild-type skin in the thickness of epidermis. Keratin 14 is a marker for undifferentiated epithelium and the basal layer of skin ectoderm (Kopan and Fuchs, 1989). It was similarly expressed in the basal epithelial cells in E14 wild-type and *Ta* embryos (Fig. 6I,J). The distribution and intensity of keratin 10, a marker of the stratified layer (Byrne et al., 1994), was similar in E15 *Ta* and wild-type skin (Fig. 6K,L). At E17, no apparent difference was seen in the thickness of the epidermis between wild-type and *Ta* mice. Filaggrin, which is expressed in granules of terminally differentiated keratinocytes, was detected by immunostaining in the back skin of both wild-type and *Ta* embryos (Fig. 6M,N). Hence, the differentiation of the keratinocytes appeared not to be affected in the *Ta* skin.

Search for downstream targets of ectodysplasin/Edar signaling: molecular analysis of hair follicles in *Ta* mutants

The expression of *Edar* in the hair placodes raises the possibility that ectodysplasin/Edar signaling may regulate their signaling activity. We compared the expression of several candidate target genes of the common signaling pathways in the placodes of E15 and E17 wild-type and *Ta* mutant embryos by radioactive in situ hybridization. In E15 wild-type embryos, *Lef1* and *Shh* showed co-expression with *Edar* in the tylotrich hair placodes of back skin ectoderm (Fig. 7A,E,I). *Lef1* was also intensely expressed in the underlying mesenchyme (Fig. 7E). Activin βA, *Bmp4* and *Ptch* showed particularly intense expression in the mesenchymal cell condensates under the placodes (Fig. 8A,E,I). Tylotrich placodes do not form in *Eda* ectoderm, and at E15 none of the genes that in the wild-type skin was confined to the placode ectoderm or underlying mesenchyme showed punctuated expression. They were either absent, e.g. *Shh*, *Ptch* and *Bmp4*, or showed expression throughout the ectoderm or underlying mesoderm, e.g. activin, *Edar* and *Lef1* (Fig. 7B,F,J; Fig. 8B,F,J). The finding that *Edar* was expressed in the ectoderm of *Ta* skin at E15 indicates that its expression does not depend on its own signaling. However, the observation that it did not show a punctuated pattern indicates that ectodysplasin/Edar signaling is upstream of the patterning of *Edar* and other placodal genes of the placodes.

At E17, when the awl follicles in *Ta* mutant skin have been initiated, all placodal genes analyzed showed intense localized expression in the ectodermal placodes and/or in the underlying mesenchyme (Fig. 7D,H,L; Fig. 8D,H,L) indicating that ectodysplasin/Edar signaling is not required for their expression in the second wave awl follicles.

To determine whether WNT family genes were expressed in *Ta* skin, and whether they are regulated by ectodysplasin/Edar signaling, we analyzed the expression patterns of *Wnt6* and *Wnt10a*. At E17, *Wnt6* was expressed in the epidermal cell layer both in wild-type and *Ta* skin (Fig. 9A,B). *Wnt10a* transcripts were seen in the basal epithelial cells in E15 and E17 wild-type and *Ta* skin, as well as in stage 3-4 tylotrich follicles in E17 wild-type skin (Fig. 9C-F).

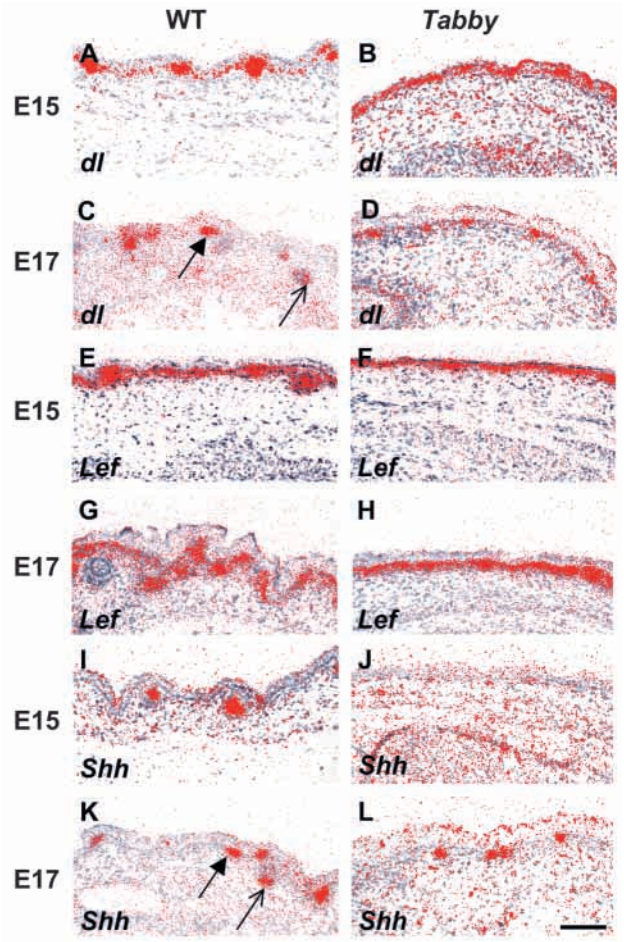

Fig. 7. Comparison of ectodermal gene expression patterns in wild-type and *Ta* mutant skin. (A,E,I) In E 15 ectoderm, *Edar* (*dl*) is expressed in the placodes of the first wave tylotrich hairs, and *Lef1* (also expressed in the underlying mesenchyme) and *Shh* show similar expression in placodes. (C,G,K) At E17, the same genes are largely co-expressed in the developing tylotrich hair follicles (skeleton arrows) and in placodes of awl follicles (arrows). (B,F,J) In *Ta* mutant skin, no placodes have been initiated at E15 and expression of *Edar*, and *Lef1* is seen throughout the ectoderm. *Lef1* is also expressed in mesenchyme. (N) *Shh* transcripts are absent in E15 *Ta* mutant skin. (D,H,L) In E17 *Ta* skin, placodes of awl follicles have been initiated, and *Edar* and *Shh* are expressed in the placode ectoderm. (L) *Lef1* expression is seen throughout the basal epithelium, in the placodes and also in underlying mesenchyme. Scale bar: 100 μm.

Because ectodysplasin/Edar signaling was recently reported to be genetically upstream of β-catenin (Huelsken et al., 2001), we analyzed the expression of β-catenin transcripts. In wild type embryos β-catenin expression resembled that of *Lef1*. It was confined to the ectoderm and underlying mesoderm at E15-E17 and was particularly intense in the bulbs of hair follicles in newborn and 2-day-old mice (Fig. 9G,I,K, and data not shown). In *Ta* mutants, expression also resembled that of *Lef1* and was intense at the epithelial mesenchymal interface at E15 and E17 and in postnatal mice it was upregulated in the hair follicles (Fig. 9L). These findings suggest that ectodysplasin/Edar signaling does not directly regulate β-catenin expression.

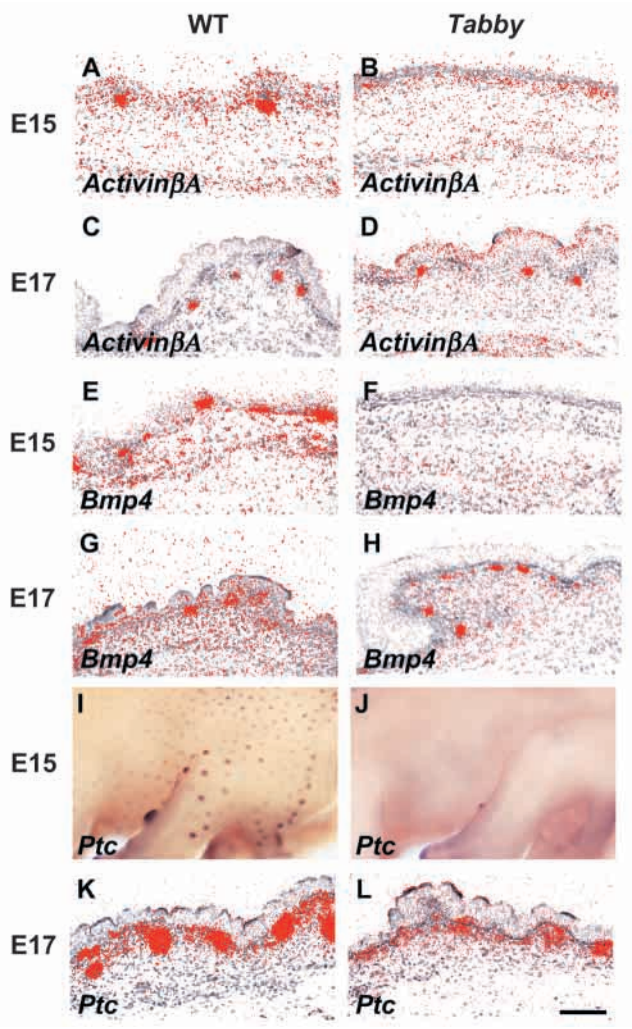

Fig. 8. Comparison of gene expression in the skin mesenchyme of wild-type and *Ta* mutant embryos. (A,E,I) Activin βA, *Bmp4* and *Ptch* are intensely expressed in the mesenchyme under the ectodermal placodes in E15 wild-type embryos. *Ptch* (*Ptc*) expression is seen in whole mount in situ hybridization. (C,G,K) In E17 wild-type embryos, activin βA, *Bmp4* and *Ptch* are expressed in the tylotrich hair follicles and awl placodes. (B,F,J) In E15 *Ta* mutants, no placodes have formed and no upregulation of gene expression is apparent. Activin βA is expressed weakly throughout the mesenchyme underlying the ectoderm. (D,H,L) At E17, activin βA, *Bmp4* and *Ptch* are intensely expressed in the *Ta* mesenchyme that underlies the placodes of awl hairs. Scale bar: 100 μm.

DISCUSSION

Ectodysplasin and Edar mediate signaling within early ectoderm and between interfollicular epidermis and hair placodes

The expression of both *Edar* and *Eda* was mainly restricted to ectodermal tissue during hair follicle development, indicating that TNF signaling takes place within epithelial tissue. *Eda* and *Edar* transcripts were detected throughout the thin ectoderm from E11 until E13. When epidermal placodes formed at E14,5, *Edar* was intensely upregulated in these early signaling centers, whereas *Eda* was excluded from these sites. Expression of *Eda* continued in the interfollicular ectoderm as demonstrated for ectodysplasin in developing human and mouse skin (Montonen et al., 1998; Mikkola et al., 1999), whereas *Edar* transcripts were downregulated in these areas (Headon and Overbeek, 1999). *Eda* and *Edar* expression

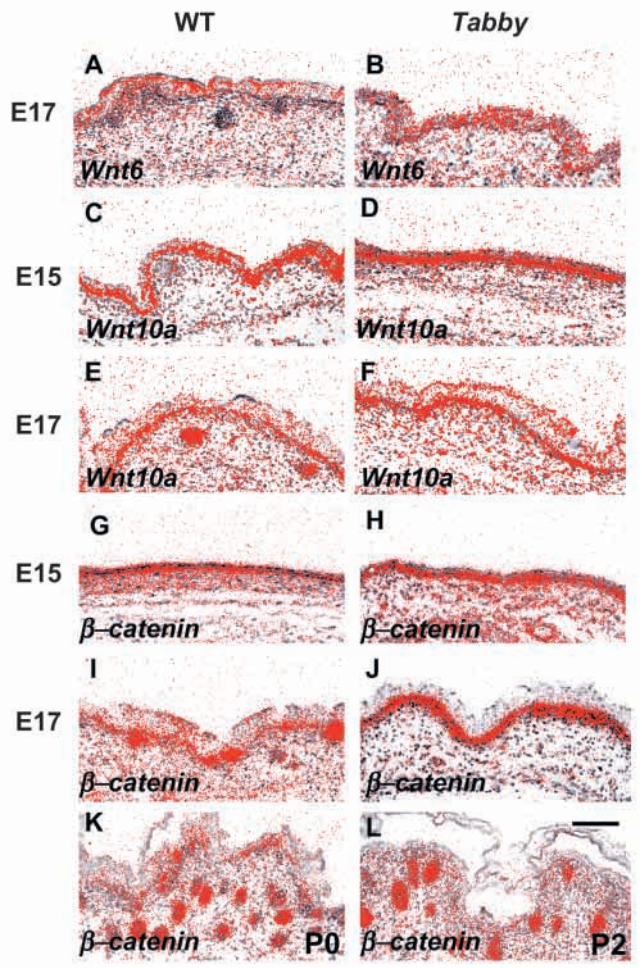

Fig. 9. Expression of *Wnt6*, *Wnt10a* and β-catenin in wild-type and *Ta* mutant embryos. (A,B) *Wnt6* is expressed in the epithelium of both wild-type and *Ta* mutant E17 skin. (C-F) *Wnt10a* transcripts are expressed in the basal epithelial cell layer in wild-type and *Ta* mutant skin as well as in hair follicles. (G,H) β-catenin is expressed in the epithelium and underlying mesenchyme in E15 wild-type and *Ta* mutant skin. (I-L) During later stages, β-catenin transcripts become strongly upregulated in the hair follicles both in wild type and *Ta* mutant skin, and expression continues in the basal epithelium as well. Scale bar: 100 μm.

subsequently colocalized in the bulb region of follicles from stage 4-5 onwards (E17).

In the hair placodes, *Edar* was co-expressed with genes of several conserved signaling pathways including *Lef1, Shh* and the gene for β-catenin (Lyons et al., 1990; Zhou et al., 1995; Bitgood and McMahon, 1995). This suggests that TNF signaling is integrated with other signal pathways and that it has roles in the formation and function of the epithelial signaling centers during hair morphogenesis. The patterns of *Eda* and *Edar* expression are strikingly similar during tooth development as *Eda* and *Edar* are first co-expressed in oral ectoderm and subsequently *Edar* localizes to show punctuated expression in ectodermal signaling centers first in the placodes initiating budding and subsequently in the enamel knots regulating tooth shape (Jernvall and Thesleff, 2000). As in skin, *Eda* is also expressed in teeth in the ectoderm outside the signaling centers (Laurikkala et al., 2001). Hence, similar observations in the two ectodermal organs suggest conserved roles for TNF signaling during ectodermal organ development. Interestingly, it was recently shown that also scale formation in fish depends on Edar signaling indicating that its function has been conserved during the evolution of organs that form as ectodermal appendages (Kondo et al., 2001).

Ectodysplasin, like other TNF ligands, is produced as a type II membrane protein. Some of these proteins are shed from the cell surface and are thus capable of diffusing to cells which are not in intimate contact (Gruss and Dower, 1995; Baker and Reddy, 1998). Ectodysplasin has a putative cleavage site containing a recognition-sequence for a furin-like enzyme, and it was shown recently that this site is required for its cleavage (Elomaa et al., 2001). It is conceivable that ectodysplasin is shed from the cells of the interfollicular ectoderm in order to reach its receptors in the placodes. Interestingly, based on phenotypic features of the skin defects in heterozygote individuals with HED as well as on results from tissue recombination studies of *Tabby* and *downless* mutant skin, it was suggested already 30 years ago that the product of *Eda* gene is a diffusible molecule (Sofaer, 1973).

Activin and dermal mesenchyme regulate *Edar* expression in epidermal placodes

Separation of the epithelial and mesenchymal tissues leads to loss of *Eda* and *Edar* expression in the skin ectoderm. Because the epithelial-mesenchymal interactions regulating hair follicle development are reciprocal and they coordinate morphogenesis at all stages of hair development, it is not possible to conclude from these experiments how direct the mesenchymal effects are on *Eda* and *Edar* regulation. However, our experiments on the effects of signal molecules on *Eda* and *Edar* expression indicated that *Edar* expression may actually be regulated by the dermal mesenchyme, because activin A was the only one of the tested signals that upregulated *Edar* expression in the skin explants. We showed that activin βA is expressed in mesenchyme underlying the ectoderm before follicle initiation, and becomes subsequently restricted to mesenchyme underlying the placodes. Our in situ hybridization analysis indicated an intricate correlation between the expression of mesenchymal activin A and placodal *Edar*.

Activin A upregulated *Edar* expression in the ectoderm only when the mesenchyme was present, and this stimulation took place specifically in the placodes (and not around the beads). This indicates that activin cannot induce *Edar* in epidermis in the absence of placode formation. Activin stimulates *Edar* expression in the epithelial signaling centers also in developing teeth (Laurikkala et al., 2001), and we showed in the present study that this induction also requires the presence of mesenchyme. In addition, in tooth the site of upregulated *Edar* expression did not depend on the location of the bead but was confined to the signaling centers at the enamel knots. Activin A knockout mice lack whiskers and most teeth, but no other hair phenotype has been reported (Matzuk et al., 1995). In tooth development, its function is required before bud stage (Ferguson et al., 1998). We showed that activin expression starts early in dermal mesenchyme, and it is possible that some of its effects are mediated by the stimulation of *Edar* expression in the epithelium. These pathways therefore seem conserved between hair and teeth. It is possible that presence of some proteins in epithelium, which are required for transduction of activin signal, e.g. its own cell-surface receptor, depends on induction by another mesenchymal signal. It is also possible that co-operation of two signals is needed for *Edar* expression, or that activin induces in the mesenchyme another signal regulating *Edar* expression. In any case, our observations indicate that TNF signaling in epithelium is regulated by mesenchymal signals and that activin may be such a signal. We propose that by upregulating *Edar* expression activin may control the responsiveness of the placodes to TNF signals from the interfollicular epidermis (Fig. 10).

WNT signals induce *Eda* expression and are transduced by LEF1

Of the tested signaling molecules, only WNT6 induced *Eda* expression in the epidermis, and our previous findings indicate that the same regulatory pathway operates in tooth development (Laurikkala et al., 2001). *Wnt6* was the only WNT family gene analyzed, and it may have mimicked the effects of other family members. *Wnt6* is expressed in the skin ectoderm with several other WNTs, and some, although fewer WNT genes are expressed in the mesenchyme (Reddy et al., 2001). *Wnt3a* and *Wnt7a* which are expressed in the epithelium, affect gene expression in the dermal component of hair follicles and induce the maintenance of hair-inducing activity of the dermal papilla (Kishimoto et al., 2000). It has been suggested that an epithelially expressed WNT may coordinate the development of epidermis and dermis during hair follicle development. It is an intriguing possibility that these WNT signals may regulate the development of the epidermal placodes by inducing *Eda* expression (Fig. 10).

The observed downregulation of *Eda* expression in the ectoderm of *Lef1*$^{-/-}$ embryos indicates that LEF1 may mediate the nuclear effects of the WNT signals regulating *Eda*. The LEF1/TCF DNA-binding proteins form a transcription factor complex with β-catenin that is translocated into the nucleus as a result of WNT signaling (Behrens et al., 1996; Willert and Nusse, 1998). The downstream targets of LEF1/TCFs have been largely unknown. The possibility that *Eda* may be a direct target of LEF1 is supported by the presence of a conserved LEF1-binding site in the promoter region of both the murine *Eda* gene and its human counterpart *ED1* (Kere et al., 1996; Srivastava et al., 1997). Co-transfection of LEF1 and β-catenin has recently been shown to increase the transcription of *ED1* (Durmowicz et al., 2002). Furthermore, a mutation has been

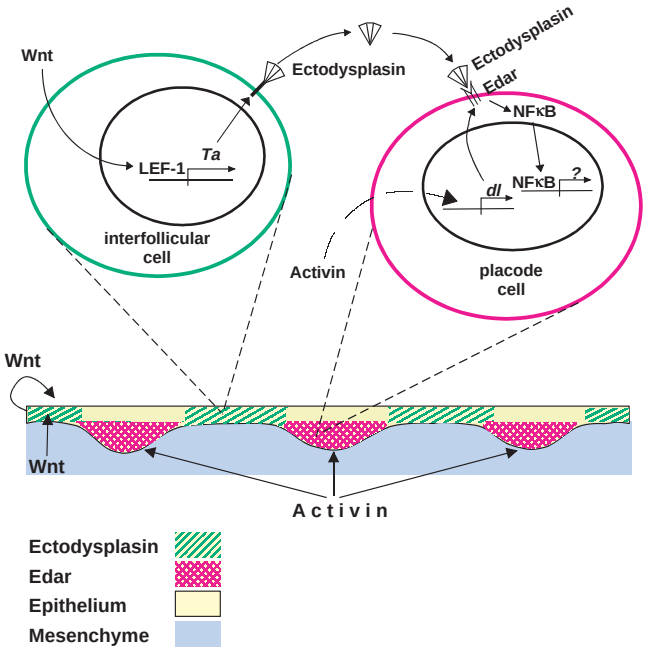

Fig. 10. The regulation of hair follicle formation by the TNF signal ectodysplasin [encoded by *Eda* (*Ta*) gene] and its receptor Edar [encoded by *Edar* (*dl*) gene], and integration with other signaling pathways. Ectodysplasin is expressed in the interfollicular ectoderm and induced by WNT signals, which are transduced by LEF1. Edar is expressed in the placodes and stimulated by activin signals from the underlying mesenchyme. Ectodysplasin is shed (secreted) and binds as a trimer to the trimerized Edar receptor. This TNF signaling is transduced by the NF-κB transcription factor.

identified in this promoter region in an individual with HED (Kobielak et al., 1998).

Although hair follicle development is inhibited in *Lef1* knockouts and *Tabby* and *downless* mutants, the hair phenotypes of the mice are different. Whiskers are fewer in number and tail hairs more affected in *Ta* and *dl* mutants than in *Lef1*$^{-/-}$ mice. Furthermore, as confirmed by our study, the first wave of hair follicle development (tylotrichs or guard hairs), which is totally absent in *Ta* mutants occurs in *Lef1* mutants, although the follicles are abnormal and reduced in number (Grüneberg, 1966; van Genderen et al., 1994). There are several possibilities to expalin these discrepancies. For example, different molecular mechanisms may regulate the development of different hair types, the different expression patterns of *Eda* and *Lef1* during initiation may account for such differences. It is also possible that *Eda* and *Lef1* signaling pathways counteract at some level, or that expression differences between genes in same families play roles. LEF1 may be redundant with other TCF-family transcription factors (Okamura et al., 1998), and ectodysplasin and Edar with other TNF ligands and receptors. We are presently analyzing these different possibilities.

Although *Edar* and *Lef1* are co-expressed in developing skin, first throughout early ectoderm and subsequently in placodes, the analysis of *Lef1*$^{-/-}$ embryos indicated that *Edar* expression does not depend on LEF1. This is in line with the finding that WNT did not stimulate *Edar* expression, and also with our previous demonstration of unaffected *Edar* expression in the signaling centers in the teeth of *Lef1* mutants (Laurikkala et al., 2001).

Ectodysplasin/Edar signaling regulates hair follicle morphogenesis but not epidermal cell differentiation

The striking expression pattern of *Edar* during hair follicle morphogenesis indicates a role for TNF signaling in the formation and/or function of epithelial placodes and hair follicles. *Edar* is expressed in the placodes and in the bulb of follicles in all types of hairs, including tylotrich (first forming) and zig zag (last forming) hairs, which are absent in the *Ta* mutants, but expression was also apparent in the placodes of awl (second forming) hairs which do develop in the *Ta* mutants. Possible explanations for the formation of awl hairs in *Ta* mice are redundancy of ectodysplasin and Edar with other TNFs and receptors, or compensation of TNF signals with other signaling pathways. There are several lines of evidence indicating that other TNF ligands and receptors exist that regulate embryonic morphogenesis and may have redundant functions with ectodysplasin and edar. A novel receptor, XEDAR (X-linked ectodysplasin-A2 receptor), which is expressed in hair follicles binds the EDA-A2 splice form of ectodysplasin, whereas EDA-A1 splice variant binds to Edar (Yan et al., 2000). Another novel TNF receptor that is expressed in hair follicles, called TROY (Tnfrsf19 – Mouse Genome Informatics) has been cloned (Eby et al., 2000; Kojima et al., 2000). Furthermore, *Eda* and *Edar* are expressed in mice and humans in a variety of tissues which are not affected in ectodermal dysplasia syndromes (Montonen et al., 1998; Mikkola et al., 1999) (J. Pispa and I. Thesleff, unpublished), suggesting that they may have redundant functions with other TNFs and TNF receptors. The broad expression patterns also suggest that the developmental regulatory functions of TNFs may not be restricted to ectodermal organs.

TNF signals are commonly mediated via the activation of NF-κB and this pathway is also used in ectodysplasin/Edar signaling (Kumar et al., 2001; Doffinger et al., 2001; Koppinen et al., 2001). NF-κB has been associated with apoptosis as well as cell survival, and we speculate that in the case of ectodysplasin/Edar, it may promote cell survival rather than apoptosis. The phenotype of *Ta* and *dl* mutants, in which the affected organs show deficient development, support this view, and we also have not detected effects in apoptosis either in cells overexpressing *Edar* nor in the enamel knot signaling centers of *Ta* mutant teeth (Koppinen et al., 2001).

We could not confirm the earlier suggestion that the differentiation of the skin is affected in *Ta* mutants (Vielkind and Hardy, 1996). All studied differentiation markers of the keratinocytes, keratin 14, keratin 10 and filaggrin, were present suggesting that ectodysplasin/Edar signaling does not essentially regulate cell differentiation in the epithelium. This is in line with several studies in which alterations of the NF-κB activity in basal epithelial cells in skin did not affect the expression patterns of differentiation-specific markers (Kaufman and Fuchs, 2000). NF-κB signaling has, however, been frequently implicated in epidermal differentiation (Kaufman and Fuchs, 2000). NF-κB activity in basal keratinocytes appears to regulate the cessation of cell cycle, as the inhibition of NF-κB activity leads to hyperproliferation of epidermis which is not seen in *Ta* mutants. It is conceivable that these later effects of NF-κB in epidermis are not regulated

by ectodysplasin/Edar signaling, as *Edar* is not expressed in the basal epithelial cells after E15 when placodes have formed.

Ectodysplasin/Edar signaling has an early function in patterning of hair placodes

We addressed the role of ectodysplasin/Edar signaling in the formation of hair placodes by analyzing hair follicle morphogenesis and gene expression patterns in the *Ta* mutant mice, which completely lack the first wave of hair follicles (tylotrich or guard hairs), whereas the second wave awl follicles do develop in the mutants. Their awl follicles expressed *Edar* together with several molecules of conserved signaling pathways. This indicated first that the expression of *Edar*, which encodes TNF receptor, does not depend on its own signaling. Secondly the results indicated that ectodysplasin/Edar signaling is not needed for the expression of the other genes analyzed in awl placodes. However, these observations do not necessarily exclude the possibility that some of the genes could be downstream targets of *Edar* because there may be a redundant TNF signal operating in the awl follicles.

The placodes of the first developing tylotrich (guard) follicles were morphologically absent in E15 *Ta* ectoderm, and *Edar*, like all other placodal genes analyzed, did not show punctated expression in presumptive locations of placodes. Therefore, it was not possible to identify targets of ectodysplasin/Edar signaling in the tylotrich follicles either. However, a recent study in which β-catenin function was conditionally deleted in the ectoderm indicates that *Edar* acts upstream of most other genes in the placodes. The development of the tylotrich follicles was inhibited, and several placodal markers including *Shh*, *Ptch* and *Bmp4* were absent. However, *Edar* was expressed in a punctate pattern resembling the distribution of hair placodes, indicating that it acts upstream of β-catenin, which could be placed upstream of the other placodal genes (Huelsken et al., 2001). We did not detect differences in β-catenin expression between wild-type and *Ta* mutant skin by in situ hybridization analysis, which suggests that ectodysplasin/Edar signaling is not needed for β-catenin expression. Hence, at present, the target genes of ectodysplasin/Edar signaling in the placodes remain unknown.

Because *Edar* expression is not patterned to the hair placodes in the *Ta* mutants, it is conceivable that ectodysplasin/Edar signaling has a role already before actual placode formation, and acts further upstream, perhaps in the patterning process regulating hair placode initiation. WNT signaling has been frequently implicated in the early inductive events during hair and feather development. For example, overexpression of TCF/LEF1 transcription factors and constitutive activation of β-catenin affects the patterning of hair follicles and stimulates their development (Gat et al., 1998; Noramly et al., 1999). Our demonstration that *Eda* expression is induced by WNT signals, which are transduced by LEF1, is intriguing. It is also conceivable that β-catenin is required for this signal transduction, although *Edar* is patterned to the placodes in the ectoderm of conditional β-catenin mutants (Huelsken et al., 2001). Because the Cre-mediated recombination in the ectoderm occurred later than the stage of placode patterning, β-catenin expression presumably was not affected during the patterning process. In conclusion, we propose that the effects of WNT signaling during the initiation of placode development are at least partly due to upregulated ectodysplasin expression, and the subsequent stimulation of TNF signaling and activation of NF-κB-responsive genes in the early ectoderm.

A reaction-diffusion model for placode patterning in feathers and hairs has been put forward, which assumes the existence of diffusible activators of placodes, such as FGFs, and inhibitors, e.g. BMPs (Jung et al., 1998; Oro and Scott, 1998). Ectodysplasin could be a key activator in such a model as has previously been suggested (Barsh, 1999). This patterning is an early event and takes place when *Eda* and *Edar* are co-expressed in the ectoderm. What are the downstream targets of this signaling and what leads to the patterned expression of *Edar* and subsequently of other placodal markers will be an interesting question to be answered in future studies.

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