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Ectodysplasin has a dual role in ectodermal organogenesis: inhibition of Bmp activity and induction of Shh expression

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Ectodermal organogenesis is regulated by inductive and reciprocal signalling cascades that involve multiple signal molecules in several conserved families. Ectodysplasin-A (Eda), a tumour necrosis factor-like signalling molecule, and its receptor Edar are required for the development of a number of ectodermal organs in vertebrates. In mice, lack of *Eda* leads to failure in primary hair placode formation and missing or abnormally shaped teeth, whereas mice overexpressing *Eda* are characterized by enlarged hair placodes and supernumerary teeth and mammary glands. Here, we report two signalling outcomes of the Eda pathway: suppression of bone morphogenetic protein (Bmp) activity and upregulation of sonic hedgehog (Shh) signalling. Recombinant Eda counteracted Bmp4 activity in developing teeth and, importantly, inhibition of BMP activity by exogenous noggin partially restored primary hair placode formation in *Eda*-deficient skin in vitro, indicating that suppression of Bmp activity was compromised in the absence of Eda. The downstream effects of the Eda pathway are likely to be mediated by transcription factor nuclear factor-κB (NF-κB), but the transcriptional targets of Edar have remained unknown. Using a quantitative approach, we show in cultured embryonic skin that Eda induced the expression of two Bmp inhibitors, Ccn2/Ctgf (CCN family protein 2/connective tissue growth factor) and follistatin. Moreover, our data indicate that Shh is a likely transcriptional target of Edar, but, unlike noggin, recombinant Shh was unable to rescue primary hair placode formation in *Eda*-deficient skin explants.

KEY WORDS: Ccn2, Ectodermal dysplasia, Lateral inhibition, NF-κB, Tabby, Mouse

INTRODUCTION

The first visible sign of ectodermal organ development is the appearance of an ectodermal placode, a local thickening of the epithelium. Formation of the placode is accompanied by condensation of the underlying mesenchymal cells (Pispa and Thesleff, 2003). Typically, placodes develop sequentially in specific patterns, such as teeth at precise locations along the dental lamina or hairs and feathers at regular intervals within the integument. Individual organ primordia need signals for their initiation, expansion and termination. It is apparent that both positive and negative regulators of placodal fate are involved in these processes, and a reaction-diffusion model has been set forth to explain the establishment of periodic patterning of hair and feather buds (Oro and Scott, 1998; Jung and Chuong, 1998; Jiang et al., 1999). Many signalling molecules (and their inhibitors), including Wnts, fibroblast growth factors (Fgfs), transforming growth factor-βs (Tgf-βs) and sonic hedgehog (Shh), are expressed in the placodes or by the underlying condensed mesenchyme (Pispa and Thesleff, 2003; Schmidt-Ullrich and Paus, 2005; Mikkola and Millar, 2006). Wnts and Fgfs are well-known promoters of placodal cell fate (Gat et al., 1998; Jung et al., 1998; Noramly et al., 1999; Huelsken et al., 2001; Andl et al., 2002), whereas bone morphogenetic proteins (Bmps) of the Tgf-β superfamily are generally regarded as placode inhibitors (Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999).

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Ectodysplasin-A (Eda), a member of the tumour necrosis factor (Tnf) superfamily is an early and necessary signal required for placode formation (for reviews, see Thesleff and Mikkola, 2002; Mikkola and Thesleff, 2003). Recent studies have indicated that the Eda pathway is downstream of the primary inductive signal required for placode initiation, yet lies high in the hierarchy of molecules positively regulating placodal cell fate (Mustonen et al., 2004). Although dental, mammary and secondary hair placodes form normally in *Eda*-deficient mice (*Tabby* mice) (Pispa et al., 1999; Laurikkala et al., 2002; Kangas et al., 2004) (M.P. and M.L.M., unpublished), mice carrying mutations in any of the necessary components of the Eda signalling pathway lack primary hair placodes giving rise to guard hairs (Mikkola and Thesleff, 2003). In humans, mutations in the *Eda* pathway genes cause hypohidrotic ectodermal dysplasia syndrome featured by missing or malformed teeth, sparse hair and the absence of a number of exocrine glands.

Many of the details in the Eda signalling pathway have been uncovered during recent years. Studies with cultured cells transfected with wild-type or mutant Edar, the receptor for the Eda-A1 isoform of ectodysplasin, have suggested that activation of the transcription factor NF-κB is crucial for Eda signalling (Yan et al., 2000; Koppinen et al., 2001; Kumar et al., 2001). In addition, phenotypic analyses of mice and humans with compromised NF-κB responses indicate that Edar signalling is mediated for most part, if not totally, by the I-κB kinase (Ikk)-dependent canonical NF-κB pathway in vivo (Schmidt-Ullrich et al., 2001; Puel et al., 2004). Recently, NF-κB reporter activity in primary hair placodes was shown to be dependent on Eda (Schmidt-Ullrich et al., 2006) in line with our own observations (M.L.M., unpublished). Thus far, the direct downstream target genes regulated by Edar have not been found.

Overexpression of Eda-A1 in developing epidermis results in supernumerary tooth and mammary placodes, which develop into mature organs. Moreover, Eda-A1 transgenic embryos are

characterized by increased placodal size, and treatment of embryonic skin with recombinant Eda-A1 in vitro promotes placodal cell fate in a dose-dependent manner (Mustonen et al., 2004). The effects of Eda-A1 are highly similar to those brought about by the best-known positive regulators of placode formation such as noggin, a potent inhibitor of Bmps (Noramly and Morgan, 1998; Botchkarev et al., 1999). Interestingly, the consequences of the ablation of noggin and *Eda* are converse to each other in terms of hair placode formation: primary hair follicle formation is dependent on Eda, whereas secondary hair follicles require noggin for initiation (Botchkarev et al., 2002).

Eda signalling also influences later stages of ectodermal organ development. Absence of *Eda* leads to an obvious molar cusp patterning defect associated with a smaller enamel knot, an epithelial signalling centre regulating tooth shape (Pispa et al., 1999). Forced expression of Eda-A1 or Edar results in a lack of enamel in incisors, which is associated with the absence of ameloblasts, the epithelial cells producing the enamel matrix (Mustonen et al., 2003; Pispa et al., 2004; Tucker et al., 2004). A similar phenotype was recently reported in mice overexpressing follistatin or noggin (Wang et al., 2004a; Plikus et al., 2005). These findings together with the similar effects of Eda-A1 and noggin on placode formation prompted us to test whether Edar activity could counteract Bmp signalling.

In this study, we provide evidence that recombinant Eda antagonizes the activity of Bmp4 in developing incisors and provide evidence indicating that suppression of Bmp activity is compromised in *Eda*-deficient skin. By using a quantitative approach, we found that the expression of Ccn2/Ctgf (CCN family protein 2/connective tissue growth factor), a multifunctional secreted protein (Perbal, 2004) known to antagonize Bmp4 activity (Abreu et al., 2002) was strongly induced by Eda-A1 in cultured embryonic skin. The expression pattern of *Ccn2* correlated with that of *Edar* in nascent hair and tooth placodes. In addition, follistatin was moderately upregulated by Eda-A1. Finally, we show that *Shh* was strongly induced by Eda-A1 in developing skin, but, unlike noggin, recombinant Shh did not rescue hair placode formation in *Eda*-null skin.

MATERIALS AND METHODS

Animals

Wild-type female mice from the NMRI strain were kept by breeding with NMRI males. The appearance of a vaginal plug was taken as embryonic day (E) 0. The maintenance and breeding of *Eda*-deficient mice (*Tabby* mice, also referred to as *Eda*-null or $Eda^{-/-}$ mice; Jackson Laboratories stock #JR 0314) has been described earlier (Pispa et al., 1999).

Organ cultures

Wild-type E15 incisors were dissected in Dulbecco's PBS pH 7.4 under a stereomicroscope. Embryonic tooth explants were grown on nuclepore filters at 37°C for 24 hours in a Trowell-type culture containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine and penicillin-streptomycin. Affi-Gel agarose beads (BioRad) were soaked in bovine serum albumin (BSA, 1 μg/μl, Sigma) or in recombinant, purified Fc-Eda-A1 protein (250 ng/μl) (Gaide and Schneider, 2003), and heparin acrylic beads (Sigma) were soaked in Bmp4 protein (100 ng/μl, R&D Systems) for 45 minutes at 37°C. The beads were placed on top of the explants using fine forceps, and explants were cultured for 24 hours. When indicated, Eda-A1 or BSA-releasing beads were introduced 6 hours before Bmp4 beads followed by a further 24 hours of culture.

In rescue experiments, back skin from carefully staged E13 *Eda*-null or wild-type embryos was dissected and cultured as previously described (Laurikkala et al., 2002). Recombinant noggin (R&D Systems) or sonic hedgehog (R&D Systems) was administered to the culture medium as indicated in the text.

In situ hybridization

Whole embryos, isolated mandibles or cultured explants were treated with cold methanol for 2 minutes, fixed in 4% paraformaldehyde overnight, and processed for whole-mount in situ hybridization as described earlier (Mustonen et al., 2004) by using the InSituPro robot (Intavis AG, Germany). The digoxigenin-labeled probes were detected with BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim Gmbh, Germany). The following plasmids were used as templates: ameloblastin (Wang et al., 2004a); β-catenin, *Edar* and *Shh* (Laurikkala et al., 2002); follistatin (Wang et al., 2004b); and a 0.8 kb probe specific to the 3′ end of *Ccn2* (Friedrichsen et al., 2003). Noggin (McMahon et al., 1998), gremlin (Khokha et al., 2001), *Dan* (Dionne et al., 2001) and bambi (Grotewold et al., 2001) probes were labelled with ^{35}S-UTP, and radioactive in situ hybridization on paraffin sections was performed according to standard procedures as described previously (Laurikkala et al., 2002).

Hanging drop cultures and quantitative RT-PCR

To analyse the induction of putative target genes by Eda-A1, tissues were grown submerged in hanging drops. E14 wild-type or $Eda^{-/-}$ back skin was dissected in Dulbecco's PBS pH 7.4 and cut in two halves along the midline: one half was used as the control and the other one was exposed to Eda-A1. A minimum of triplicate samples was assayed each time. Skin-halves were placed in culture medium and allowed to recover in a cell culture incubator for about 30 minutes. When grown in the absence of serum, MEM was supplemented with glutamine, 0.2% bovine serum albumin and 20 mmol/l Hepes, pH 7.2. Each skin half was cultured individually in one drop of 40 μl pre-warmed medium supplemented with Eda-A1, or equivalent proportion of protein dissolvent, placed under the lid of a 35 mm diameter plastic Petri dish containing medium or PBS to prevent evaporation (James et al., 2006).

Tissues from hanging drops (or freshly isolated E14 wild-type or K14-Eda-A1 skin) were placed straight into 350 μl lysis buffer of the RNeasy mini kit (Qiagen) containing 1% β-mercaptoethanol (Sigma). Total RNA was isolated as specified by the manufacturer and quantified using UV spectroscopy. One hundred to 700 ng of total RNA was reverse transcribed using 500 ng of random hexamers (Promega) and 100 units of Superscript II (Invitrogen) according to the manufacturer's instructions. Quantitative PCR (qPCR) was carried out using the 2× SYBR-green PCR master mix (Applied Biosystems) and Applied Biosystems' default PCR conditions for the ABI 7000 as described (James et al., 2006). Primer sequences are available upon request. PCR products were run on a 2% agarose gel to verify their correct size and the absence of non-specific reaction products and primer dimers. Gene expression was quantified by comparing the sample data against a dilution series of PCR products (amplicons) of the gene of interest. Data were analysed using Applied Biosystems' Prism SDS software and normalized against *Ranbp1*.

Promoter analysis

The mouse and human promoter sequences of *Ccn2*, follistatin and *Shh* genes were aligned with LALIGN, and analysed for the presence of putative NF-κB binding sites by Match and P-Match programs that are freely available on the Internet.

RESULTS

Ectodysplasin-A1 counteracts Bmp4 activity in the developing mouse incisor

In rodent incisors, only the labial side (anterior side) of the tooth is covered by enamel, while the lingual side (facing tongue) is devoid of it. Mice overexpressing Eda-A1, follistatin or noggin share similar incisor phenotype, i.e. they lack ameloblasts and therefore also enamel (Mustonen et al., 2003; Wang et al., 2004a; Plikus et al., 2005). We showed recently that the K14-follistatin phenotype results from suppression of Bmp activity by ectopic follistatin expressed in the labial side of the incisor, thereby leading to the inhibition of ameloblast differentiation. In cultured incisor explants, recombinant follistatin antagonized Bmp4-induced expression of the ameloblast-specific gene ameloblastin (Wang et al., 2004a). Due to the obvious similarity of the three transgenic phenotypes, we considered the

incisor culture as a useful system to monitor Bmp activity and to test whether Eda-A1 can suppress it. In accordance with our previous results, Bmp4-releasing beads induced intense ameloblastin expression (5/5 explants) compared with control explants, which displayed the typical expression pattern on the labial side of the incisor epithelium (Fig. 1A,B). However, unlike follistatin (Wang et al., 2004a), Eda-A1-releasing beads placed next to a Bmp4 bead did not affect the expression of ameloblastin in the majority of the explants (5/7 explants) (Fig. 1D), although occasionally a prominent reduction was detected (2/7 explants; data not shown), which we never observed with BSA-soaked beads (Fig. 1C). However, it is unlikely that the Tnf family protein Eda could interfere with Bmp activity by directly binding to Bmps and/or binding to Bmp receptors. Its effects, if any, would more probably be indirect and might require induced expression of a Bmp antagonist. In order to test this possibility, we introduced beads soaked with Eda, or control beads releasing BSA, 6 hours before a Bmp4 bead. Remarkably, we noticed a near total absence of ameloblastin expression in explants pretreated with Eda (24/27 explants), whereas BSA had no effect (21/23 explants) (Fig. 1E,F). Eda also appeared to reduce the endogenous level of ameloblastin mRNA to a certain extent (compare Fig. 1G with 1A). These results demonstrate that activation of the Edar pathway is able to counteract Bmp4 signalling and suggest a plausible explanation for the observed enamel phenotype of K14-*Eda* and K14-*Edar* mice (Mustonen et al., 2003; Pispa et al., 2004; Tucker et al., 2004).

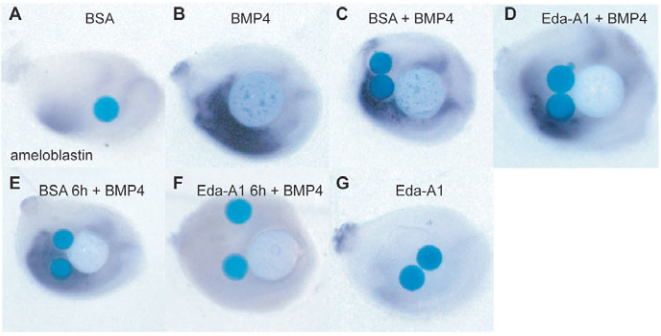

Fig. 1. Eda-A1 suppresses BMP activity in developing incisors. Whole-mount in situ hybridization analysis of ameloblastin expression in E15 incisors cultured with protein-releasing beads for 1 day. (**A**) Some endogenous expression of ameloblastin is present in explants cultured with a BSA bead. (**B**) Ameloblastin was strongly induced by a Bmp4-releasing bead. (**C**) BSA-releasing beads (small and blue) were placed at the same time with the Bmp4 bead (large and white). (**D**) Eda-A1 beads (blue) had no major effect on Bmp4-induced expression of ameloblastin when applied simultaneously with the Bmp4 bead (white). (**E**) BSA beads (blue) applied 6 hours before the Bmp4 bead (white) had no effect on ameloblastin expression. (**F**) Introduction of Eda-A1-releasing beads (blue) 6 hours before the BMP bead (white) strongly inhibited the induction of ameloblastin. (**G**) The endogenous expression of ameloblastin was reduced in the presence of Eda-A1 (compare with A).

Search for the physiological targets of Edar

A putative physiological target of Edar should have an overlapping expression pattern in developing teeth and/or hair follicles (see also below). During tooth development, *Edar* becomes restricted to dental placodes as they form (E12) (Fig. 2A) (Tucker et al., 2000; Laurikkala et al., 2001). At the bud stage (E13), expression of *Edar* was intense at the tip of the tooth bud, and at the cap stage (E14) it was confined to the enamel knot, an epithelial signalling centre regulating tooth shape (Fig. 2B). Similarly, during hair development *Edar* is first detected throughout the epithelium, becomes localized to nascent placodes, and is later most intense at the tip of the growing hair follicle (Headon and Overbeek, 1999; Laurikkala et al., 2002).

Extracellular high-affinity antagonists of Bmps include, among others, noggin, gremlin (gremlin 1, *Grem1* – Mouse Genome Informatics), Dan (Neuroblastoma, *Nbl1* – Mouse Genome Informatics), chordin, follistatin, ectodin (scelerostin domain containing 1, *Sostdc1* – Mouse Genome Informatics), and Ccn2/Ctgf (also known as Fisp12) (Balemans and Van Hul, 2002). The expression profiles during tooth and/or hair development of only some of these have been previously described. Therefore we analysed the expression of a number of Bmp inhibitors between E12 and 16. Noggin is expressed in the mesenchymal condensate under the epithelial hair placode (Botchkarev et al., 1999), but we found no expression in the developing dental placode, although intense expression was observed in Meckel's cartilage (Fig. 2C). Only faint expression of noggin was detected at later developmental stages (Fig. 2D, and data not shown). Expression of gremlin and *Dan*, two Bmp inhibitors with similar structural motifs and inductive activities (Balemans and Van Hul, 2002), was limited to the mesenchyme of the developing tooth at E12 (Fig. 2E,G), and in subsequent developmental stages (Fig. 2F,H, and data not shown). Expression of gremlin is confined to the interplacodal mesenchyme during feather development (Ohyama et al., 2001). Interestingly, follistatin is expressed in the tooth, hair and feather placodes (Ferguson et al., 1998; Patel et al., 1999; Nakamura et al., 2003), and is localized to the enamel knot of E14 molar teeth (Wang et al., 2004b). Also *Ccn2*, a modular multifunctional protein with known ability to inhibit Bmp signalling (Abreu et al., 2002), is expressed in the epithelium of developing teeth, and is localized to the enamel knot of the cap stage tooth and is found later in preameloblasts (Shimo et al., 2002; Friedrichsen et al., 2003; Yamaai et al., 2005). Although ectodin, a modulator of Bmp and Wnt pathways, is mainly epithelial, its expression domain does not significantly overlap with that of *Edar* (Laurikkala et al., 2003).

Bambi is a pseudoreceptor related to the Tgf-β superfamily type I receptors and negatively regulates Bmp, activin and Tgf-β signalling (Balemans and Van Hul, 2002). At the placode and bud stage it was expressed in the condensed mesenchyme (Fig. 2I, and data not shown), while at the cap stage prominent expression was also evident in the epithelium overlapping the enamel knot region (Fig. 2J). Smad6 and Smad7 are inhibitory Smads, which antagonize the Tgf-β pathway, binding either to the type I receptor or Smad4 (Derynck and Zhang, 2003). Interestingly, *Smad7* transcripts have been detected in the epithelium of developing teeth (Luukko et al., 2001), and the expression of this gene can be regulated by NF-κB (Derynck and Zhang, 2003). In conclusion, based on the published data and our own expression analyses, we considered follistatin, Ccn2, bambi and Smad7 as best candidates mediating the Bmp-inhibitory action of Eda-A1.

Noggin is able to partially restore hair placode formation in *Eda*-null embryos

Eda-deficient mice lack primary hair follicles and the localized expression of a battery of placode markers at E14 (Laurikkala et al., 2002). Bmps have a well-established role as placode inhibitors during feather and secondary hair placode formation (for reviews, see Millar, 2002; Schmidt-Ullrich and Paus, 2005), and at least *Bmp4* and *Bmp7* are expressed in developing murine skin, and their uniform expression is retained in E13 and 14 *Eda*-deficient skin (data not shown). Therefore we reasoned that one crucial outcome of Eda signalling during hair

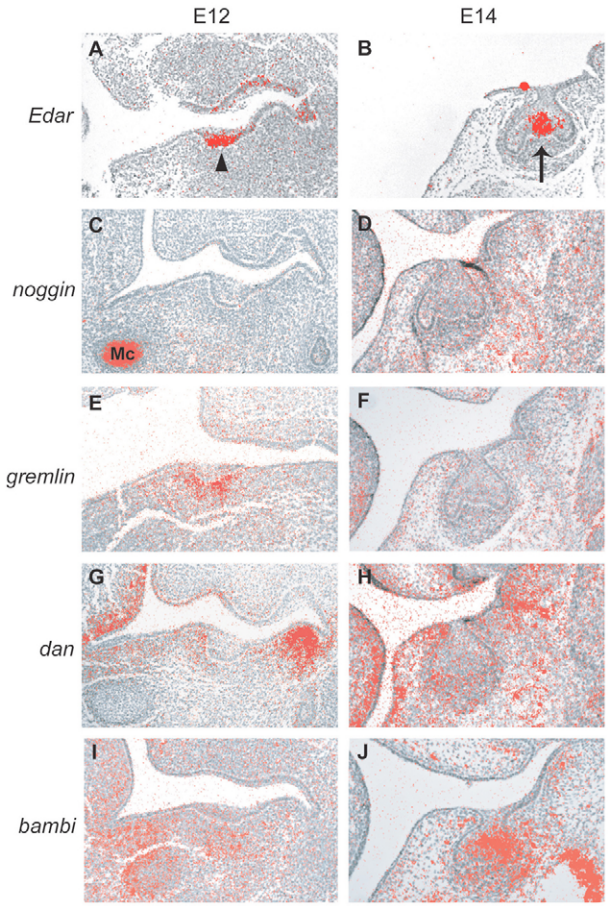

Fig. 2. Expression patterns of *Edar* and BMP inhibitors in wild-type molar teeth. In situ hybridization with probes specific to *Edar* (**A**,**B**), noggin (**C**,**D**), gremlin (**E**,**F**), *Dan* (**G**,**H**) and bambi (**I**,**J**) at the placode stage (A,C,E,G,I) and at the cap stage (B,D,F,H,J) of tooth development. *Edar* is expressed in the dental placode (arrowhead) at E12, and in the enamel knot (arrow) at E14. Noggin, gremlin and *Dan* were detected only in the mesenchyme, whereas bambi was also expressed in the epithelium overlapping the enamel knot at E14.

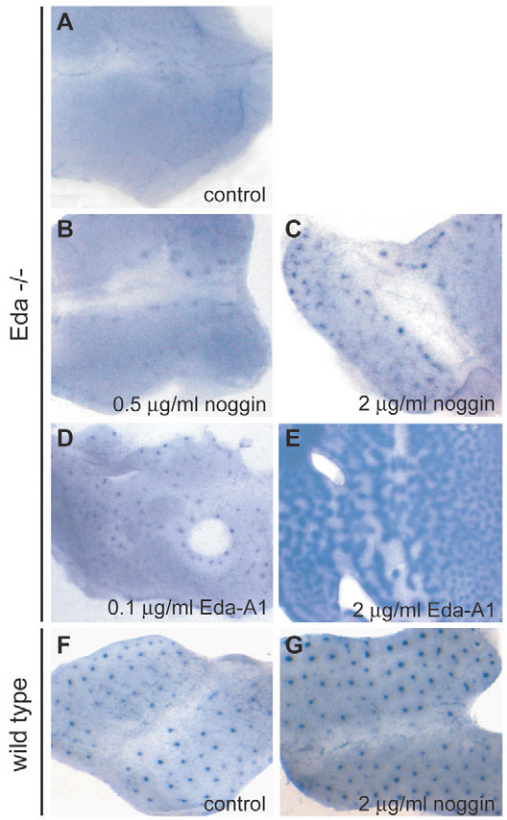

Fig. 3. Noggin partially restores hair placode formation in *Eda*-deficient skin. E13 skin explants were cultured for 1 day and hair placode induction was detected by expression of placode marker *Shh*. (**A**) *Eda*-deficient explants cultured in the control medium lack the primary hair follicles. (**B**,**C**) Explants cultured in the presence of 0.5 μg/ml exogenous noggin (B) partially restored hair placode induction, whereas a more prominent rescue was seen with 2 μg/ml of noggin (C). (**D**,**E**) Eda-A1 at 0.1 μg/ml restored the expression of *Shh* (D), whereas 2 μg/ml Eda-A1 caused a prominent enlargement of placodes in *Eda*$^{-/-}$ skin (E). (**F**,**G**) *noggin* at 2 μg/ml caused enlargement of hair placodes in wild-type skin (G) compared with untreated explants (F).

placode formation could be suppression of Bmp activity. If this was the case it might be possible to restore primary hair placode in *Eda*-deficient mice by an exogenous Bmp inhibitor. Low doses of recombinant Eda-A1 induce normally sized and spaced placodes in E13 *Eda*$^{-/-}$ skin explants cultured for 24 hours, whereas high doses of Eda-A1 cause enlargement and fusion of placodes (Mustonen et al., 2004) (Fig. 3D,E). Treatment of E13 *Eda*$^{-/-}$ skin explants with 0.5 μg/ml of recombinant noggin induced the formation of some placodes (Fig. 3A,B), whereas 2 μg/ml of noggin led to the development of multiple placodes seen as a more prominent punctuate expression of placode-specific genes throughout the explant (Fig. 3C). In wild-type skin, noggin slightly increased the size and number of hair placodes (Fig. 3F,G), in line with previous reports (Noramly and Morgan, 1998; Botchkarev et al., 1999). Interestingly, the spacing of noggin-rescued follicles of Eda-deficient skin was not as regular as that seen in untreated wild-type skin or in explants rescued by low doses of Eda-A1 (Fig. 3D,F), and we never noticed as prominent enlargement of placodes as seen with superfluous Eda-A1 (Fig. 3E) (Mustonen et al., 2004). These results strongly suggest that lack of primary hair placode formation in *Eda*-null mice is at least partly due to insufficient inhibition of Bmp activity, but that additional Eda targets are likely to be involved.

Eda induces the expression of *Ccn2* and follistatin in skin explants

To assess whether any of the candidate genes (see above) is regulated by Edar, we used a novel approach that combines skin explant culture with quantitative analysis of immediate responses to recombinant Eda. *Eda*$^{-/-}$ back skin was isolated at E14, i.e. at the time when primary hair placodes form in wild-type embryos. Each skin explant was divided into two halves along the dorsal midline and cultured with or without Eda in a hanging drop to ensure rapid and uniform distribution of the recombinant molecule. This setup allows us to analyse the induction kinetics of a gene of interest and thereby distinguish primary effects from the secondary ones. Upon exposure to Eda-A1, expression of follistatin, *Ccn2*, bambi and *Smad7* was monitored by quantitative RT-PCR.

In the first set of experiments, Eda-A1 was applied in a culture medium containing 10% serum and the expression of the candidate genes was analysed at 1 hour intervals (1-6 hours) (Fig. 4A). We observed a rapid increase in the levels of *Ccn2* transcripts, being 2-fold at 1 hour, peaking at around 3-4 hours (about 11-fold) and gradually decreasing after that. We also noticed a moderate but consistent 2-fold increase in the amount of

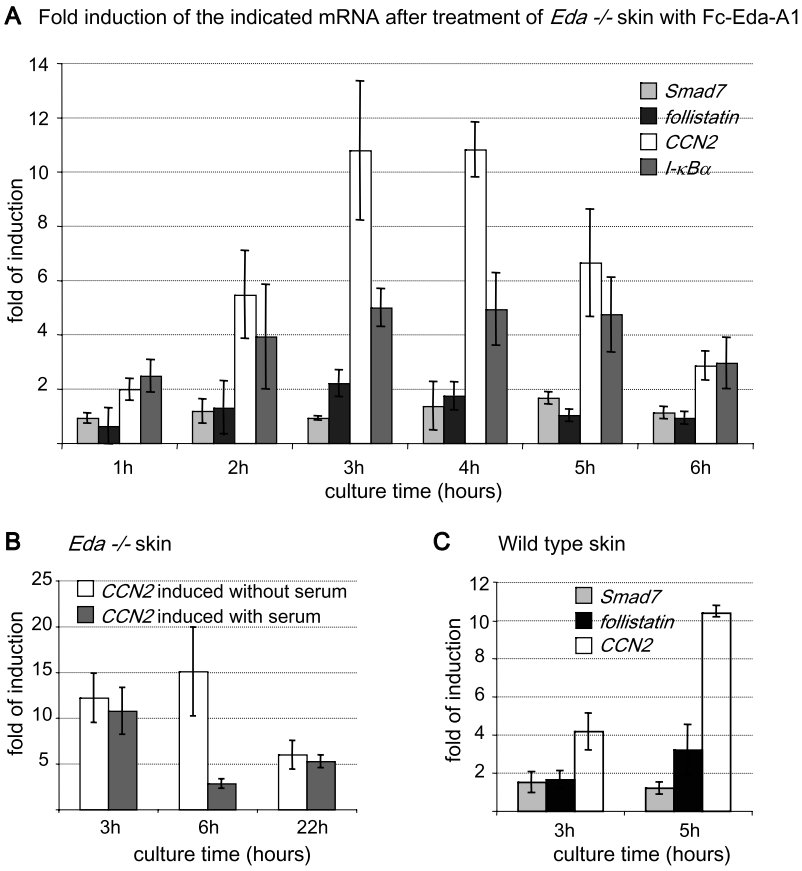

Fig. 4. Expression of *Ccn2* and follistatin is induced by Eda-A1. *Eda*-deficient (**A**,**B**) or wild-type (**C**) E14 individual skin explants were cut in two halves along the midline and cultured in the absence or presence of 2 μg/ml Eda-A1, and gene expression was analysed by quantitative RT-PCR. (A) A timecourse of expression of *Ccn2*, *Smad7*, follistatin and *I-κBα* in *Eda*-deficient skin upon Eda-A1 stimulus. Upregulation of *Ccn2* is detected already after 1 hour of Eda treatment and peaks at 3-4 hours. *I-κBα* is induced with kinetics similar to *Ccn2*. (B) Comparison of *Ccn2* induction by Eda-A1 in skin explants cultured in a medium with or without serum. *Ccn2* transcripts are maintained at high level for a longer time in the absence of serum. (C) Induction of *Ccn2*, *Smad7* and follistatin gene expression in wild-type skin after exposure to Eda-A1 in the absence of serum.

follistatin after 3 hours exposure to Eda-A1, whereas there were no noticeable changes in the level of *Smad7* and bambi within the time interval tested (Fig. 4A and data not shown). These results demonstrate that *Ccn2*, and possibly follistatin, are downstream of Eda-A1 in embryonic skin.

The rapid induction of *Ccn2* suggested that it might be a direct transcriptional target of Edar, most likely regulated by NF-κB activation (see Introduction). To correlate the kinetics of *Ccn2* expression with that of a validated NF-κB target gene, we also analysed the expression of *I-κBα* in the same samples (Scott et al., 1993; Hoffmann et al., 2003). I-κBα is an inhibitor of NF-κB, and its expression is induced by a number of Tnf receptors, thereby participating in a feedback loop of NF-κB activity (Hayden and Ghosh, 2004). Expression of *I-κBα* correlates with that of *Edar* and NF-κB activation in developing molars (Laurikkala et al., 2001; Ohazama et al., 2004). Consistently, its localized expression in primary hair placodes is dependent on Edar (Schmidt-Ullrich et al., 2006). Expression of *I-κBα* was induced to about 2.5-fold after 1 hour exposure to Eda-A1, was highest (about 5-fold) at 3-4 hours and slowly declined thereafter (Fig. 4A).

Next, we performed a similar series of experiments in the absence or presence of serum (Fig. 4B) in order to eliminate the contribution of serum-derived factors in our experimental set-up, as *Ccn2* has been identified as one of the immediate-early genes induced by serum growth factors (Rachwal and Brigstock, 2005). After 3 hours of exposure to Eda-A1, *Ccn2* expression was highly induced (about 10-fold) under both conditions. At 6 hours, a 15-fold induction of *Ccn2* was detected in the absence of serum, whereas again a decrease to about 2.5-fold was seen in the presence of serum. When the actual numbers of *Ccn2* transcripts induced by 6 hour treatment of Eda-A1 were compared, the difference between the two samples was less prominent (data not shown). The reason for this is that in control explants (no Eda-A1 added) there was more *Ccn2* in the presence of serum, and therefore the fold of induction was lower (data not shown). However, about twice as many *Ccn2* transcripts were induced by Eda-A1 at 6 hours in the absence of serum, suggesting that a serum component may inhibit Eda-A1 activity. After 22 hours of culture, a sustained level of *Ccn2* expression (about 5-fold) was detected in both culture conditions (Fig. 4B).

Finally, we tested the ability of Eda-A1 to induce *Ccn2*, follistatin and *Smad7* in wild-type E14 skin explants (Fig. 4C). Like in *Eda*-deficient skin, we noticed no effect in the expression of *Smad7* upon Eda-A1 treatment, whereas a 4-fold and 10-fold augmentation in *Ccn2* levels was observed at 3 hours and 5 hours, respectively. The fold of induction was slightly lower than in *Eda*-deficient skin (Fig. 4B), mainly due to the fact that the initial amount of *Ccn2* transcripts was higher in wild-type skin (data not shown). qPCR analysis of epithelia separated after 3 hours exposure to Eda-A1 confirmed that *Ccn2* was specifically induced in the epithelium (data not shown). A 3-fold induction in the expression levels of follistatin was evident at 5 hours of culture with Eda-A1 (Fig. 4C). A modest increase of *Ccn2* transcripts was also observed in K14-Eda-A1 skin at E14 compared with non-transgenic littermates (data not shown).

Expression of *Ccn2* correlates with that of *Edar* during early stages of pelage hair and tooth development

Currently, only limited knowledge on the expression profile of *Ccn2* during ectodermal organ development is available (Shimo et al., 2002; Friedrichsen et al., 2003; Yaamai et al., 2005). Therefore, we analysed the expression of *Ccn2* by whole-mount in situ hybridization during early hair and tooth development (Fig. 5). At

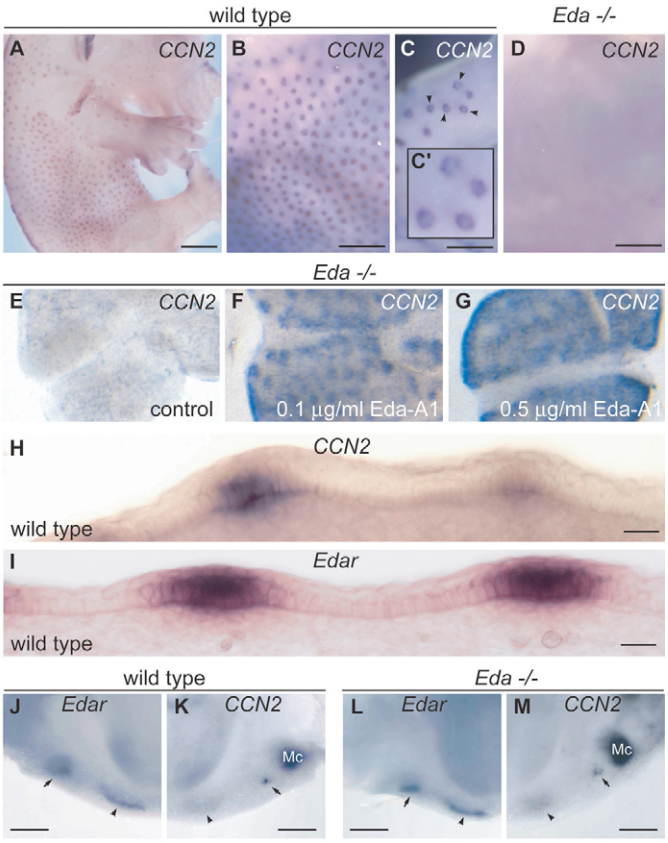

Fig. 5. *Edar* and *Ccn2* colocalize during early hair and tooth development. Whole-mount in situ hybridization with a probe specific to *Ccn2* (**A-G**,**H**,**K**,**M**) and *Edar* (**I**,**J**,**L**). (A-C′) *Ccn2* is expressed in primary hair placodes of wild-type embryos at E14, and is often concentrated at the circumference of the placode (arrowheads in C). (D) No localized expression of *Ccn2* is detected in *Eda*-null skin at E14. (E-G) Recombinant Eda-A1-induced localized upregulation of *Ccn2* in $Eda^{-/-}$ E13 skin explants cultured for 24 hours. (H,I) Vibratome section of whole mounts confirmed the colocalization of *Edar* and *Ccn2* in primary hair placodes of E14 wild-type embryos. (J,K) *Edar* and *Ccn2* are coexpressed also in wild-type molar (arrow) and incisor (arrowhead) placodes at E12. (L,M) In contrast to primary hair placodes, expression of *Ccn2* is unaffected in *Eda*-deficient tooth placodes. Mc, Meckel's cartilage. Scale bar: 1 mm in A; 0.5 mm in B-D,J-M; 50 μm in H,I.

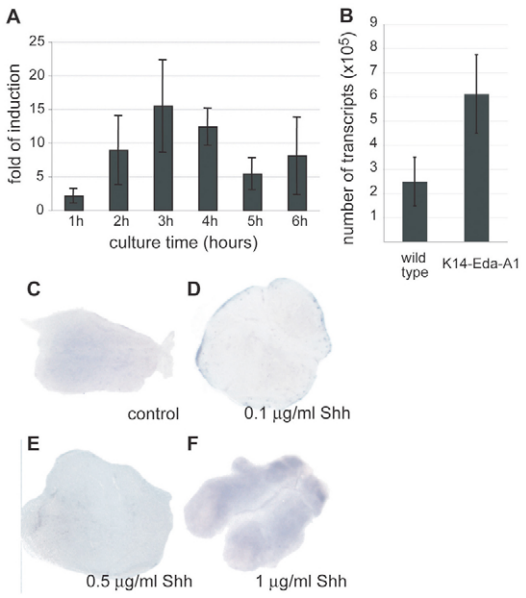

Fig. 6. Eda-A1 upregulates transcription of *Shh*, but recombinant Shh is unable to rescue hair placode induction in *Eda*-deficient skin. (**A**) *Eda*-deficient skin explants were cultured in the absence or presence of Eda-A1, as depicted in Fig. 4A, and *Shh* expression was analysed by quantitative RT-PCR. A 2.5-fold induction of *Shh* was evident already after 1 hour of treatment, and was highest after 3-4 hours of treatment. (**B**) The number of *Shh* transcripts was increased in E14 *K14-Eda-A1* skin compared with control littermates. (**C-F**) *Eda*-deficient E13 skin explants cultured for 1 day in the control medium are devoid of hair follicles and no rescue is detected with 0.1 μg/ml, 0.5 μg/ml or 1.0 μg/ml recombinant Shh.

E14, *Ccn2* was expressed in nascent primary hair placodes of wild-type embryos (Fig. 5A,B), whereas the epithelium of the developing mystacial vibrissae of the snout was devoid of *Ccn2* (Fig. 5A) (Friedrichsen et al., 2003). Interestingly, closer examination of pelage hair follicles revealed that *Ccn2* was sometimes concentrated at the periphery of placodes (Fig. 5C,C′). *Ccn2* expression was detected also in the cartilage of the digits as previously described (Fig. 5A) (Friedrichsen et al., 2003). No localized expression of *Ccn2* was detected in the skin ectoderm of *Eda*-null embryos at E14 (Fig. 5D), although expression in the digits was unaltered (data not shown). However, treatment of $Eda^{-/-}$ skin explants with recombinant Eda-A1 induced localized upregulation of Ccn2 in placodes (Fig. 5E-G and data not shown). Vibratome sections of the whole mounts of wild-type embryos confirmed the colocalization of *Ccn2* and *Edar* in nascent hair placodes (Fig. 5H,I).

Finally, we compared the expression of *Ccn2* and *Edar* during early tooth development. At E12, *Edar* was confined to incisor and molar placodes of wild-type embryos (Fig. 5K), as formerly shown by radioactive in situ hybridization (Tucker et al., 2000; Laurikkala et al., 2001). Likewise, expression of *Ccn2* was detected in both molar and incisor placodes in addition to a prominent signal in Meckel's cartilage (Fig. 5L). In *Eda*-deficient embryos, *Edar* transcripts were fairly normally distributed (Fig. 5M), as demonstrated previously during a more advanced stage of molar development (Tucker et al., 2000). Interestingly, and in contrast to what was seen in skin, there was no gross difference in the expression of *Ccn2* between wild-type and *Eda*-null E12 mandibles (Fig. 5N).

Shh is also a likely target of Edar but does not rescue hair placode formation in *Eda*-null skin

Although noggin was able to restore hair placode formation in *Eda*-null skin, its effects differed from those of recombinant Eda-A1 on the spacing and size of placodes (Fig. 2) (Mustonen et al., 2004). It is possible that this was due to improper amount, location or timing in the application of exogenous noggin, but it may also suggest that suppression of Bmp activity is not sufficient to mimic all of the effects of Eda-A1. We have previously shown that *Shh* transcripts are detected upon rescue of *Eda*-null skin with overnight treatment of recombinant Eda (Mustonen et al., 2004). Moreover, *Shh* is coexpressed with *Edar* in developing hair follicles, and in embryonic teeth from bud stage onwards (Bitgood and McMahon, 1995; Laurikkala et al., 2001; Laurikkala et al., 2002). These data prompted us to analyse whether *Shh* is a target of Edar. Strikingly, a 2.5-fold induction of *Shh* was evident already after 1 hour treatment of E14 skin-halves with Eda-A1, and by 3 hours it had increased to 15-fold (Fig. 6A). The overall *Shh* induction displayed kinetics

highly similar to those of *Ccn2* and *I-κBα* (Fig. 4A). The amount of *Shh* in K14-Eda-A1 whole skin samples was increased 2.5-fold compared with control littermates (Fig. 6B). In mice lacking *Shh*, hair follicle formation is initiated and Shh is thought to regulate proliferation and downgrowth of the follicular epithelium (St-Jacques et al., 1998; Chiang et al., 1999). In line with the proposed later role of Shh in follicular morphogenesis, we found no indication of placode formation in $Eda^{-/-}$ skin explants treated with recombinant Shh protein (Fig. 6C-F).

DISCUSSION

In this study we report two novel functions for the Edar signalling pathway: inhibition of Bmp activity and positive regulation of *Shh* expression. Expression of *Ccn2*, also known as connective tissue growth factor, a multifunctional protein known to antagonize Bmp activity (Abreu et al., 2002), *Shh* and to a lesser extent follistatin, was rapidly induced in embryonic skin explants upon Eda-A1 treatment. Noggin partially restored primary hair placode formation in $Eda^{-/-}$ embryos, indicating that suppression of Bmp activity was compromised in embryonic skin in the absence of Eda. Moreover, recombinant Eda antagonized the activity of Bmp4 in incisor explants. These actions are likely to be mediated by Ccn2, possibly together with follistatin. We do not exclude the possibility that Eda may also regulate Bmp activity by other means, e.g. via Tak1 (Tgf-β activated kinase 1; Map3k7 – Mouse Genome Informatics), which is thought to mediate Ikk activation by Edar (Morlon et al., 2005). Although in some cell types Tak1 acts synergistically with Bmps, it may also dramatically antagonize Bmp signalling, possibly through its effects on subcellular localization of R-Smads (Hoffmann et al., 2005).

While this paper was under review, the Headon group reported sporadic rescue of hair follicle formation in noggin-treated $Eda^{-/-}$ skin (Mou et al., 2006). The more noticeable placode formation obtained by us (Fig. 2) might be due to the earlier onset of the rescue experiment (E13 versus E14) or the higher noggin concentration used (2 versus 1 μg/ml). In agreement with our results, Mou et al. (Mou et al., 2006) noticed upregulation of *Ccn2* expression by recombinant Eda-A1, as well as suppression of Bmp4-dependent Smad1/5/8 phosphorylation in Eda-A1 treated $Eda^{-/-}$ skin.

To our knowledge, *Ccn2* and *Shh* are the first likely bona fide transcriptional targets of the Eda signalling pathway discovered. The maximal fold of induction of *Ccn2* and *Shh* after pertinent Eda treatment was about 11- and 15-fold in cultured skin explants, respectively, but due to the presence of the mesenchyme (which lacks Edar and is therefore unresponsive to Eda-A1), these figures are likely to be underestimates. The rapid induction of the two genes evident already after 1 hour's exposure to Eda and the similar kinetics of I-κBα expression strongly suggest that they are direct transcriptional targets of Edar mediated via NF-κB. Comparison of mouse and human *Ccn2* promoters revealed a conserved putative binding site of NF-κB (see Table 1 in the supplementary material) (Blom et al., 2002), which is practically identical with the validated binding sites in I-κBα and M-CSF promoters [see Hoffmann et al. (Hoffmann et al., 2003) and references therein]. Furthermore, *Ccn2* colocalized with *Edar* in nascent hair placodes and in tooth germs.

Many of the actions of Ccn2 are thought to be mediated via Bmps and Tgf-β, such that it inhibits Bmp and enhances Tgf-β signalling (Abreu et al., 2002). It is likely that during hair placode formation Ccn2 has a role as a Bmp antagonist. As Ccn2 was originally isolated as a chemotactic factor for fibroblasts (Rachwal and Brigstock, 2005), it is tempting to speculate that it might also be involved in condensation of dermal cells during placode formation, a process likely to result from cell migration, rather than proliferation, as suggested by studies in chick (Wessells, 1965; Desbiens et al., 1991). Targeted gene disruption has revealed the importance of Ccn2 for chondrogenesis (Ivkovic et al., 2003). The ectodermal phenotype of *Ccn2*-null mice, which die shortly after birth, has not been described.

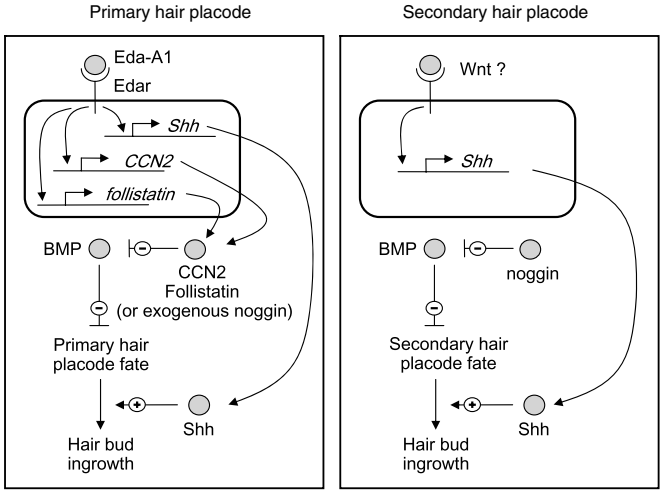

Fig. 7. A schematic representation of the outcomes of Edar activation in primary hair follicles compared to signalling in secondary hair follicles. Upon ligand engagement, Edar activates NF-κB, resulting in the upregulation of *Ccn2*, follistatin and *Shh* expression. We suggest that Ccn2 and follistatin act locally to inhibit Bmps expressed in the epithelium and/or the condensed mesenchyme, thereby allowing the expansion of the nascent placode. After the placode stage, upregulation of Shh promotes invagination of the hair bud. In secondary hair follicles, noggin expressed in the condensed mesenchyme suppresses Bmp activity within the placode, whereas other signals, possibly Wnts, induce the expression of Shh.

In addition to *Ccn2*, *Shh* was rapidly induced upon application of Eda. The ability of noggin to rescue *Shh* expression in the absence of Eda suggests that *Shh* expression is repressed, either directly or indirectly, by Bmps. In wild-type skin, the derepression could be achieved by Eda-induced Bmp antagonists, Ccn2 and follistatin, expressed in emerging hair placodes. However, the similar expression kinetics of *Ccn2* and *Shh* upon Eda treatment (Figs 4, 6) also suggests direct regulation of *Shh* expression by Eda. Analysis of *Shh* promoter sequences (–5000 to 0) revealed two putative NF-κB recognition sequences, in which the essential nucleotides were conserved between mouse and human (see Table 1 in the supplementary material). In addition, sites not conserved but with 100% match to the consensus sequence were identified in both species (data not shown).

Our qPCR data revealed a low, yet reproducible, induction of follistatin upon Eda treatment, suggesting that besides activin (Ferguson et al., 1998; Wang et al., 2004a), Edar may also regulate follistatin expression in vivo. We identified a number of putative NF-κB-binding sites in mouse and human follistatin promoters, and a conserved one (see Table 1 in the supplementary material) was located in the middle of a ~60 nucleotide region of 100% identity in mouse and human. Studies in chicken have suggested that follistatin locally antagonizes the action of the Bmps, thereby permitting feather placode formation and regulating the size of the bud (Patel et al., 1999). Hair follicle morphogenesis is retarded in follistatin knockout mice (Nakamura et al., 2003), yet primary hair follicle formation appears to be unaffected (M. Suomalainen and I.T., unpublished).

A key question is to what extent these novel functions of the Eda pathway can explain the observed phenotypes of *Eda/Edar*-deficient and *Eda*-overexpressing mice. As the molecular mechanisms involved in the early stages of development of distinct ectodermal organs are shared to a great extent (Pispa and Thesleff, 2003; Mikkola and Millar, 2006), it is possible that the same target genes are induced by Eda in different epithelial appendages. However, genes with similar functions may be induced by other signalling pathways in teeth and mammary glands but not in primary hair placodes, thereby explaining the appendage-specific phenotypes of *Eda*-null mice. The aberrant development of $Eda^{-/-}$ molars is evident from bud stage onwards and results in few shallow cusps associated with an overall smaller size of teeth (Mikkola and Thesleff, 2003; Kangas et al., 2004). Intriguingly, ablation of *Shh* (Dassule et al., 2000) and follistatin (Wang et al., 2004b) leads to small teeth with fewer cusps.

The molecular mechanism causing the supernumerary teeth and mammary glands of *K14-Eda* mice has remained enigmatic. The role of lateral inhibition, and its underlying molecular mechanism, in regulating the spacing and number of teeth and mammary glands is poorly understood, and it is not known whether Bmp signalling is involved. Instead, Shh signalling is required for tooth development already at an early stage (Hardcastle et al., 1998). The development of the ectopic molar of K14-Eda mice is marked by a *Shh*-expressing placode, and in wild-type embryos a weak and transient upregulation of *Shh* is occasionally detected in the same location (Kangas et al., 2004). We propose that the extra Shh signal produced upon increased Edar activity is crucial for promoting the development of this rudimentary dental placode into a fully erupted tooth in K14-*Eda-A1* mice. Intriguingly, in $Tg737^{orpk}$ mice carrying a hypomorphic mutation in *polaris* (*Ift88* – Mouse Genome Informatics), a regulator of Shh pathway (Haycraft et al., 2005), a supernumerary tooth develops at the same position (Zhang et al., 2003). Further studies will be required to reveal other crucial downstream components of Eda signalling in tooth and mammary development.

Previous studies have established a role for the Eda pathway as an important activator of primary hair placode fate, downstream of the yet unknown 'first dermal signal' (Mustonen et al., 2004; Houghton et al., 2005; Schmidt-Ullrich et al., 2006). During feather and hair follicle development, Bmp activity is thought to mediate lateral inhibition, such that Bmps expressed in the nascent placode prevent surrounding cells from adopting a follicular fate. Simultaneously, the action of Bmps needs to be counteracted within the placode itself (Oro and Scott, 1998). Apparently, in *Eda*-deficient embryos, no Bmp inhibition takes place and Bmps repress the follicular fate to such an extent that primary hair placodes are not discernible, whereas with increasing Edar signalling (such as that seen in *K14-Eda-A1* mice or in skin explants treated with excessive Eda) rising amounts of Bmp inhibitors are expressed, thereby allowing expansion of placodes. Our findings reveal the need for suppression of Bmp activity within nascent primary hair placodes and thereby highlight the mechanistic similarities in the induction of all pelage hair types. The fact that Eda induces Bmp antagonists other than noggin also explains why primary hair placodes are unaffected in $noggin^{-/-}$ embryos (Botchkarev et al., 2002).

Taken together, our results provide a model for the crucial role of Edar activity during primary hair placode initiation (Fig. 7). First, Eda restricts Bmp signalling in the placode by local upregulation of Bmp inhibitors. Second, Eda may regulate proliferation and ingrowth of the hair follicle through *Shh* expression, an action that is, however, required only after placode initiation (St-Jacques et al., 1998; Chiang et al., 1999). During secondary hair initiation (Fig. 7), noggin antagonizes local Bmps, whereas Wnt/β-catenin signalling is the best candidate as inducer of *Shh* (Schmidt-Ullrich and Paus, 2005; Mikkola and Millar, 2006). During early stages of tooth development, *Shh* induced by Eda together with other still unknown signals promotes the growth of the dental bud (Fig. 7). The next goal is to investigate to what extent the two signalling outcomes of ectodysplasin reported here can explain the other ectodermal defects resulting from altered Eda signalling and which other pathways are directly influenced by Edar activity.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02708/DC1

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