

Ectodysplasin A1 promotes placodal cell fate during early morphogenesis of ectodermal appendages

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Accepted 23 July 2004

Development 131, 4907-4919

Published by The Company of Biologists 2004

doi:10.1242/dev.01377

Summary

Organs developing as appendages of the ectoderm are initiated from epithelial thickenings called placodes. Their formation is regulated by interactions between the ectoderm and underlying mesenchyme, and several signalling molecules have been implicated as activators or inhibitors of placode formation. Ectodysplasin (Eda) is a unique signalling molecule in the tumour necrosis factor family that, together with its receptor Edar, is necessary for normal development of ectodermal organs both in humans and mice. We have shown previously that overexpression of the Eda-A1 isoform in transgenic mice stimulates the formation of several ectodermal organs. In the present study, we have analysed the formation and morphology of placodes using *in vivo* and *in vitro* models in which both the timing and amount of Eda-A1 applied could be varied. The hair and tooth placodes of K14-Eda-A1 transgenic embryos were enlarged, and extra placodes developed from

the dental lamina and mammary line. Exposure of embryonic skin to Eda-A1 recombinant protein *in vitro* stimulated the growth and fusion of placodes. However, it did not accelerate the initiation of the first wave of hair follicles giving rise to the guard hairs. Hence, the function of Eda-A1 appears to be downstream of the primary inductive signal required for placode initiation during skin patterning. Analysis of BrdU incorporation indicated that the formation of the epithelial thickening in early placodes does not involve increased cell proliferation and also that the positive effect of Eda-A1 on placode expansion is not a result of increased cell proliferation. Taken together, our results suggest that Eda-A1 signalling promotes placodal cell fate during early development of ectodermal organs.

Key words: Ectodysplasin, Hair placode, Tooth placode, Mammary placode, Mouse

Introduction

A number of organs in vertebrates develop as appendages of embryonic ectoderm. The morphogenesis of these organs such as hairs, feathers, teeth and mammary glands is governed by sequential and reciprocal inductive interactions between two types of tissues, the epithelium of ectodermal origin and the mesenchyme. The mesenchyme can be derived either from the mesoderm (e.g. mammary gland and hair) or from the neural crest (e.g. tooth). Despite the diversity in their mature form and function, ectodermal organs share several common features during the early steps of development. Organogenesis begins with the appearance of local epithelial thickenings, placodes, which is followed by condensation of the underlying mesenchymal cells. In the next step, the placode develops into a bud growing into or out of the mesenchyme ensued by further growth and morphogenesis typical to each organ (Pispá and Thesleff, 2003). Although the growth of the bud is generally considered to be driven by proliferation, the cellular mechanisms governing the initial steps of placode formation have not been systematically analysed. An unanswered key question is whether placode formation is primarily a cell migration-driven event or whether it rather reflects a locally

restricted burst of epithelial cell proliferation (Magerl et al., 2001; Pispá and Thesleff, 2003; Veltmaat et al., 2003). Evidently, these processes are associated with changes in cell shape as well as in their adhesive properties (Jamora et al., 2003).

In most ectodermally derived organs, the mesenchyme seems to supply the first signal required to initiate organogenesis, whereas the epithelium responds according to its inherent capabilities. Classic tissue recombination experiments using mouse and chick embryonic skin from different body parts have revealed that an initial signal(s) arising in the dermis causes the formation of placodes in epidermis from non-hair bearing regions (Hardy, 1992). Similarly, mesenchyme from mouse mammary region is able to induce functional mammary epithelium when recombined with dorsal epidermis (Veltmaat et al., 2003). The issue of whether tooth initiation is determined by neural crest derived mesenchyme or by the ectoderm is controversial (Mina and Kollar, 1987; Mitsiadis et al., 2003).

An epithelial placode can be considered a basal unit of ectodermal organogenesis. A characteristic feature of epithelial placodes is that many signal molecules including Wnts, bone

morphogenetic proteins (Bmps), fibroblast growth factors (Fgfs), and sonic hedgehog (SHH) are expressed by the placodes themselves, or by the underlying condensed mesenchyme (Millar, 2002; Thesleff and Mikkola, 2002a; Pispá and Thesleff, 2003; Veltmaat et al., 2003). These molecules are shared by many ectodermal organs, and recent studies have revealed that they regulate the initial stages of morphogenesis in these organs (Pispá and Thesleff, 2003). Therefore, it is tempting to assume that they also play identical roles, i.e. regulate similar cellular mechanisms in the initiation of all appendage types.

Ectodysplasin (Eda), a recently characterised member of the tumour necrosis factor (TNF) ligand superfamily, has also been implicated in the early development of ectodermal appendages (Barsh, 1999; Thesleff and Mikkola, 2002b; Mikkola and Thesleff, 2003). Mice deficient for Eda (*Tabby* mice), its receptor Edar (*downless* mice) or the intracellular adapter protein Edaradd that is required for Edar signalling (*crinkled* mice) have identical phenotypes characterised by defective hair development (Falconer et al., 1951; Claxton, 1967; Headon and Overbeek, 1999; Headon et al., 2001; Laurikkala et al., 2002; Yan et al., 2002). Other abnormalities of these mice include missing or misshapen teeth, as well as defects in a number of exocrine glands, such as sweat and salivary glands (Grüneberg, 1965; Sofaer, 1969; Grüneberg, 1971; Blecher et al., 1983; Pispá et al., 1999). Mutations in the corresponding human genes result in a malformation syndrome called hypohidrotic ectodermal dysplasia (HED) (Kere et al., 1996; Monreal et al., 1999; Headon et al., 2001). The symptoms of HED are similar to those found in mice, including missing or sparse hair, abnormal dentition and absent or reduced sweating (Kere and Elomaa, 2002).

Accumulating evidence suggests that Eda is an early and necessary signal required for placode formation (for reviews, see Mikkola and Thesleff, 2003; Pispá and Thesleff, 2003). First, *Tabby* mice lack primary hair follicles that give rise to guard hairs as well as the placodes for auchene and zigzag hairs (Claxton, 1967; Laurikkala et al., 2002). None of the placode marker genes tested displays a patterned expression in *Tabby* or *downless* mice at E14–E15, when the first hair follicles are forming in wild-type mice (Headon and Overbeek, 1999; Andl et al., 2002; Laurikkala et al., 2002). Second, Edar is one of the earliest markers of newly formed placodes. During early development, Eda and Edar are colocalised in the simple ectodermal sheet covering the embryo (Tucker et al., 2000; Laurikkala et al., 2001; Laurikkala et al., 2002). Upon initiation of tooth and hair development, Edar becomes restricted to the forming placodes, whereas Eda shows complementary expression in the flanking tissue. In transgenic mice that lack epithelial β -catenin, the development of hair follicles is inhibited (Huelsenken et al., 2001). In these mice Edar, but no other placode marker tested, retains its punctuate placodal expression pattern suggesting that Edar is relatively high in the hierarchy of genes regulating placodal fate. Edar is also expressed in the newly formed mammary gland placodes (Pispá et al., 2003). However, the number of mammary glands is normal in *Tabby* mice but the nipple morphology is altered (Mustonen et al., 2003). In addition, HED is occasionally associated with absent or rudimentary nipples (Clarke et al., 1987).

Third, we have recently shown that transgenic mice

overexpressing the Eda-A1 isoform of ectodysplasin (ligand of Edar) under keratin 14 (K14) promoter, which drives the expression to the developing ectoderm as early as embryonic day 9 (E9), well before the development of any ectodermal appendage is initiated, are featured by supernumerary ectodermal organs (Mustonen et al., 2003). Most notably, these mice have extra mammary glands and teeth. Furthermore, hair follicles are produced continuously between E14 and birth in K14-*Eda-A1* mice, unlike in wild-type mice where they develop in three separate waves giving rise to the different pelage hair types. In the current study, we have analysed in more detail the dynamics of ectodermal placode formation in K14-*Eda-A1* transgenic, in Eda-deficient *Tabby* and in wild-type mice, as well as in organ culture. Our results suggest that Edar signalling regulates cell fate decisions by promoting placodal fate. Epithelial placodes of K14-*Eda-A1* transgenic embryos were enlarged, and exogenous Eda-A1 stimulated the growth and fusion of placodes also in vitro. This function of Eda-A1 appears to be downstream of the initial primary inductive signal required for placode initiation during skin patterning and appears to involve cellular mechanisms other than increased proliferation of placodal cells.

Materials and methods

Animals

The generation and maintenance of K14-*Eda-A1* transgenic mice in FVB/N background has been previously described (Mustonen et al., 2003). Typically, wild-type females were mated with transgenic males, and embryos were genotyped either by PCR or by whole-mount in situ hybridisation using an Eda specific probe (Laurikkala et al., 2001; Mustonen et al., 2003). In PCR, the forward primer was specific to the β -globin intron 5'-ACATCCTGGTCATCATCCTGCC-3' and the reverse primer was specific to Eda-coding sequence 5'-GATCTTCTCCCGGTCCAAAG-3' that gives rise to a 585 bp band. The Eda-deficient *Tabby* mice were of strain B6CBACa-A^{w-J}/A-Ta, and they were obtained from Jackson Laboratories (Bar Harbor, USA) (stock #JR 0314). The maintenance of *Tabby* mice was as previously described, and all the embryos displayed the *Tabby* phenotype (Pispá et al., 1999).

Histology and in situ hybridisation

Tissues from mouse embryos were dissected and processed for in situ hybridisation as previously described (Mustonen et al., 2003). Radioactive in situ hybridisation on paraffin sections was performed according to Wilkinson and Green (Wilkinson and Green, 1990). Patched 1 (Kim et al., 1998), *Bmp4* (Vainio et al., 1993) and *Lef1* (Travis et al., 1991) probes were labelled with ³⁵S-UTP. Whole-mount in situ hybridisation analysis of embryonic day 11 (E11) to E14 whole embryos, and E11 to E13 lower jaws was carried out as described earlier (Kettunen and Thesleff, 1998) using the InsituPro robot (Intavis, Germany). The following plasmids were used as templates: β -catenin (Laurikkala et al., 2002), Edar (Laurikkala et al., 2001), *Lef1* (Travis et al., 1991), *Pitx2* (Dassule and McMahon, 1998) and sonic hedgehog (Vaahtokari et al., 1996). For plastic histology, tissues were embedded in Historesin as specified by the manufacturer (Leica) and stained using Haematoxylin and Eosin.

Scanning electron microscopy (SEM)

Embryonic mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 for at least 12 hours. Embryos were dehydrated in graded series of 50%, 70%, 94% ethanol for 1 hour each, and 100% ethanol for 12 hours, and subjected to crucial point drying and platinum coating. The back skin of shoulder regions was

viewed with Zeiss DSM 962 scanning electron microscope (Zeiss, Oberkochen, Germany).

Expression vectors

Expression construct for the ectodomain of murine Eda-A1 (amino acids 180 to 391) in pSecTag vector (Invitrogen) has been previously described (Koppinen et al., 2001). The tags of the vector were eliminated due to the usage of the authentic Eda-A1 stop codon; instead a 6× His tag was added at the N-terminal end of the recombinant protein. Plasmid coding for a single amino acid mutation in Eda-A1 coding sequence (Y343C) which abolishes receptor binding without affecting trimer formation (Schneider et al., 2001) was made by *in vitro* mutagenesis using the overlap extension method (Ho et al., 1989). Both constructs were verified by sequencing.

Production of recombinant Eda-A1 and Eda-A1 (Y343C)

Cos7 cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. For the production of recombinant proteins, ~100,000 Cos7 cells were plated on 50 mm plates. The following day, semiconfluent cells were transfected with empty pSecTag vector, Eda-A1 or Eda-A1 (Y343C) expression vectors using the Fugene 6 reagent, according to the instructions of the manufacturer (Roche Molecular Biochemicals). After 5 hours, a fresh culture medium was changed, and the cells were allowed to grow for further 24 or 48 hours before the medium was collected and used for organ culture experiments. A sample of the conditioned medium was taken and analysed for the production of recombinant molecules by western blotting (Koppinen et al., 2001) using anti-penta-his antibody as recommended (Qiagen) or antibody specific to the COOH-terminal end of Eda (Elomaa et al., 2001). Production of Fc-Eda-A1 has been previously described (Gaide and Schneider, 2003).

Organ cultures

Back skin of E12-14 wild-type or *Tabby* embryos was dissected in Dulbecco's PBS; pH 7.4 under a stereomicroscope. Skin explants were grown for 24-72 hours on nucleopore filters at 37°C in a Trowell type culture containing DMEM supplemented with 10% fetal calf serum, glutamine and penicillin-streptomycin. When indicated, conditioned medium from Eda-A1 or Eda-A1 (Y343C), or vector transfected cells was added to maximum one-fifth of the total volume. In some experiments, recombinant purified Fc-Eda-A1 (Gaide and Schneider, 2003) was added to the culture medium to a final concentration of 0.05-5.00 µg/ml. When indicated, Affigel-Blue agarose beads soaked in three concentrations (20, 75 or 500 µg/ml) of Bmp4 (R&D Systems) were placed on top of the explant.

Analysis of tooth placode size

The lower jaws of Eda-A1 transgenic embryos and their wild-type litter mates were processed for whole-mount *in situ* hybridisation using a probe specific to *Shh* to visualise the tooth placodes. Specific care was taken in determination of the age of individual embryos. After staining, the lower jaws were photographed with a digital camera along with a 1 mm grid that was used as a standard. The mesio-distal length and the linguo-

buccal width of the molar placodes were measured using NIH Image software. The measurements were visualised as box plots, the height of the box showing 50% and bars 90% of the results, median is shown as a horizontal line inside the box. Altogether, 23 molar placodes from transgenic embryos and 18 placodes of their wild-type littermates derived from two litters were analysed. For statistical analysis, the non-parametric Mann-Whitney U-test was chosen because the size of the placodes did not seem to be normally distributed. The tests were performed using JMP computer software.

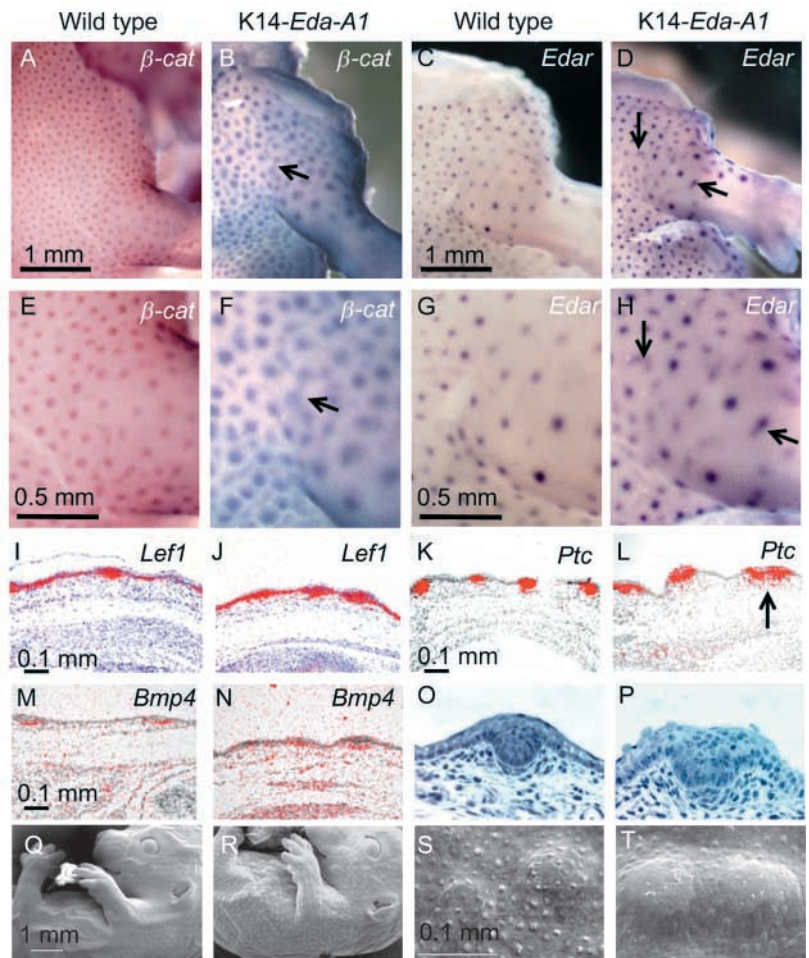


Fig. 1. Eda-A1 overexpression leads to increased size and irregular shape of the early hair placodes. Placodes were larger in K14-Eda-A1 transgenic embryonic E14 skin than in wild-type skin, as visualised by whole-mount *in situ* hybridisation by marker genes β -catenin (A,B) and *Edar* (C,D). E-H are higher magnification views of A-D. Fusions (arrow in B and F) and the irregular shape (arrows in D and H) of the early hair follicles were common in transgenic skin. *In situ* hybridisation analysis, the expression of *Lef1* was seen throughout wild-type (I) and transgenic (J) skin. Placodal epithelial and mesenchymal marker *Ptc1* also showed expansion of the hair follicles in the transgenic skin (arrow in L) when compared with wild type (K). Expression of *Bmp4* mRNA localised to the mesenchyme under the forming follicles in wild-type (M) and transgenic (N) skin. Enlarged hair follicles with irregular shape in the transgenic ectoderm (P) were evident in thin plastic sections of the E14 skin, whereas wild-type follicles were smaller and had a regular round shape (O). Scanning electron microscopy images also revealed irregularities in shape and increased sizes of the hair placodes of the transgenic embryos (R) and fusions of their follicles (T), when compared with the wild-type embryos with smaller regular placodes (Q,S).

In vivo cell proliferation assay

Two FVB/N mice mated with a K14-*Eda-A1* transgenic male were injected i.p. at E14 with 600 μ l of 5'-bromo-2'-deoxyuridine (BrdU) labelling reagent (Zymed). After 2 hours labelling, the mice were sacrificed and the embryos collected and fixed in 4% paraformaldehyde overnight. Samples were dehydrated in ethanol series and embedded in paraffin wax. Four wild-type and four transgenic embryos were cut into 7 μ m sections at the mid-back skin area. BrdU incorporation was visualised by antibody-streptavidin-biotin-peroxidase system (Zymed) and background tissue was stained with Haematoxylin. BrdU-positive and negative cells were counted under 200 \times magnification (Olympus Provis). Two-thousand five-hundred and eighty-four transgenic interfollicular cells, 3441 wild type interfollicular cells, 1937 transgenic placodal cells (40 placode sections) and 818 wild-type placodal cells (26 placode sections) were counted. The mitotic index was the proportion of BrdU-positive cells of all cells in a microscopic view or in the section of an epithelial hair placode. The *P* values were obtained using Student's *t*-test with the statistical program SPSS.

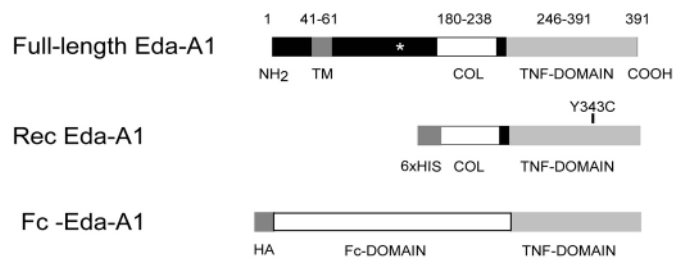
Results

Mice overexpressing *Eda-A1* have enlarged hair placodes

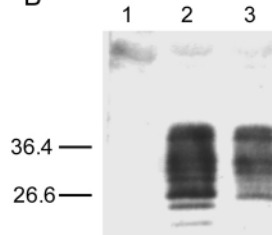
We have recently produced transgenic mice overexpressing *Eda-A1* under the keratin14 (K14) promoter (Mustonen et al., 2003). The embryonic hair phenotype of these mice is characterised by continuous production of new hair follicles abnormally close to the pre-existing ones, whereas in wild-type mice hair follicles develop in three separate waves around E14, E17 and birth (Fraser, 1951; Mann, 1962). In addition, fusions of hair follicles were seen in the K14-*Eda-A1* mice. To further analyse the pathogenesis behind the K14-*Eda-A1* phenotype, we have performed a detailed analysis of the primary hair placodes of the transgenic mice.

First, we analysed the first wave of follicles by several placodal marker genes by in situ hybridisation at embryonic day 14 (E14), a time point when the placodes have just been formed. It has recently been shown that mRNA of the ubiquitously expressed β -catenin is strongly upregulated in the epithelium of the newly formed placodes and K14-Cre mediated deletion of the β -catenin gene causes failure of placode development (Huelsen et al., 2001). Whole-mount in situ hybridisation with β -catenin probe revealed the normal hexagonal pattern of forming follicles on wild-type skin at E14 (Fig. 1A,E). Conversely, in *Eda-A1*-overexpressing embryos, the follicles appeared enlarged, their shapes were more irregular and fusions between placodes were observed (Fig. 1B,F). *Edar* mRNA encoding the receptor specific to *Eda-A1*, retained its normal placodal expression in transgenic skin but again the placodes had irregular appearance (Fig. 1C,D,G,H). The mRNA for transcription factor *Lef1*, which is crucial for hair follicle induction (van Genderen et al., 1994), is upregulated both in the epithelium and the condensed mesenchyme of the placodes (Kratochwil et al., 1996). Like β -catenin, *Lef1* mRNA was expressed within the enlarged placodes, as evidenced by both sectional (Fig. 1I,J) and whole-mount (data not shown) in situ hybridisation. Sonic hedgehog (*Shh*) is expressed in the epithelium and patched 1 is expressed both in the epithelium and in the underlying condensed mesenchyme; in *Shh*-deficient mice, hair follicles fail to develop beyond the plug stage (stage 2) (Chiang et al., 1999).

A



B



C

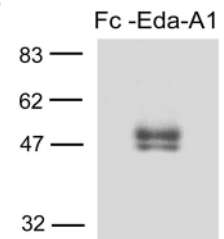
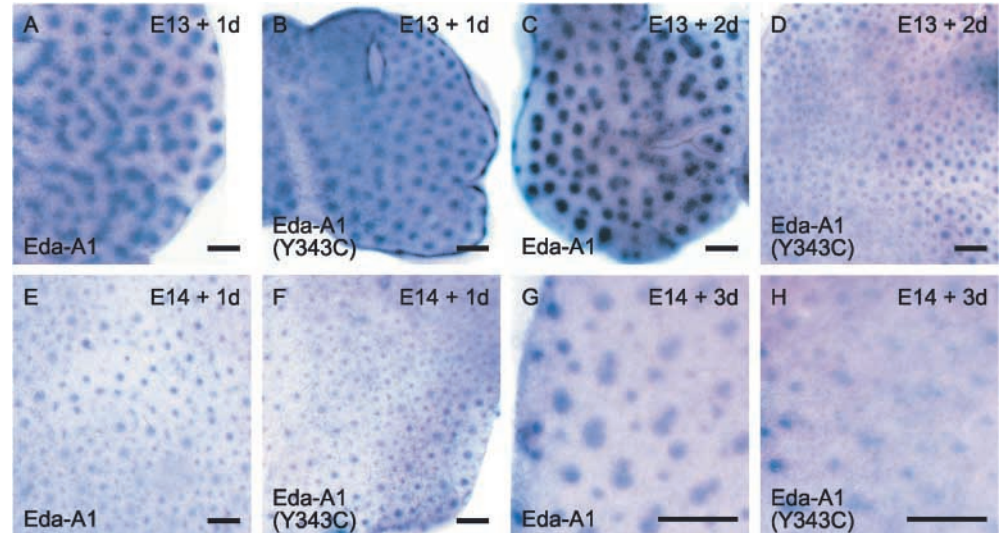


Fig. 2. Production of recombinant *Eda-A1* proteins. (A) Schematic drawing of the *Eda-A1* protein with a transmembrane domain (TM), a furin cleavage site (asterisk), collagen like Gly-X-Y repeats (COL) and the globular TNF homology domain (TNF-DOMAIN). Figures refer to amino acid numbering of endogenous *Eda-A1*. Recombinant *Eda-A1* starts at amino acid residue 180 and contains an N-terminal 6 \times His tag. Control protein contained Y343C mutation that abolishes receptor binding. Recombinant Fc-*Eda-A1* consists of an N-terminal HA tag, an Fc-domain and the TNF-domain of *Eda-A1*. (B) Cos cells were transfected with the soluble, truncated form of wild-type Rec *Eda-A1* (lane 2) or mutated Y343C *Eda-A1* construct (lane 3), or were mock transfected (lane 1). Cell supernatants were analysed by western blot using an anti-*Eda* polyclonal antibody. (C) Purified Fc-*Eda-A1* (5 μ g) was analysed by SDS-PAGE under reducing conditions and stained with Coomassie Blue. The doublet band results from glycosylation. Molecular weight markers are indicated on the left.

Expression of patched 1 is positively regulated by *Shh* and thus, reflects the signalling activity of the epithelium (St-Jacques et al., 1998; Chiang et al., 1999). Sectional in situ hybridisation with a probe specific for patched 1 revealed that the domain of the condensed mesenchyme beneath the transgenic placodes was also enlarged (Fig. 1K,L). The expression of *Bmp4*, a well-characterised inhibitor of hair and feather placodes, was also detected in transgenic embryos (Fig. 1M,N). Thus, all placode markers tested revealed the enlarged placodes of K14-*Eda-A1* embryos, indicating that more epidermal cells had adopted follicular fate.

The quality of histology was further enhanced by plastic sections of newly formed placodes (E14). These data clearly show the altered morphology of the placodes in transgenic embryos (Fig. 1O,P). To further verify the irregular pattern of primary hair placodes of *Eda-A1*-overexpressing mice, we performed scanning electron microscopy (SEM) analysis of E14 transgenic embryos in comparison with wild-type littermates. In accordance with our in situ and histological data, transgenic placodes appeared enlarged and they sometimes

Fig. 3. Eda-A1 recombinant protein increased the size and caused fusions of the hair follicles in *in vitro* cultures of embryonic skin. Hair follicles of the cultured back skin samples were analysed by whole-mount *in situ* hybridisation with β -catenin probe. E13 skin explants showed bigger and fused hair follicles after 1 day in culture with the conditioned media from cells producing recombinant Eda-A1 protein (A), compared with normal hair follicles seen in cultures with Y343 control media (B). Similar effect was seen also after two days of culture (C,D). Older explants of E14 skin showed no effects after 1 day in culture with Eda-A1 medium (E): the hair follicles were of a similar size to those of the control cultures (F). After 3 days of culture with Rec Eda-A1 media (G), the hair follicles were clearly bigger than in control cultures (H).



fused with each other (Fig. 1Q-T). As the level of transgene expression is highly upregulated already at E10 (Mustonen et al., 2003), we also analysed E13 and E13.5 embryos, but found no indication of precocious initiation of hair development by SEM nor by *in situ* hybridisation with placode-specific probes (data not shown). The counting of placodes of E14 whole-mount skin explants suggested that Eda-A1 did not increase the number of primary placodes either (data not shown).

Eda-A1 promotes enlargement of placodes in a dose-dependent manner

The enlarged guard hair placodes of Eda-A1 transgenic embryos and the absence of primary hair placodes in *Eda-* and *Edar-*deficient mice (Headon and Overbeek, 1999; Laurikkala et al., 2002) suggest that Edar regulates their initiation and/or growth. To gain further insight into the role of Eda-Edar signalling on placode formation, we performed a series of *in vitro* experiments with cultured skin explants in the absence or presence of exogenous recombinant Eda-A1. Under culture conditions hair follicles are formed essentially as *in vivo*, i.e. primary placodes are detected at E14, and secondary placodes giving rise to awl hairs at E16-E16.5.

Endogenous Eda is initially produced as a trimeric membrane-bound protein but a soluble signalling molecule, consisting of a collagenous domain followed by the TNF domain, is released by furin cleavage (Chen et al., 2001; Elomaa et al., 2001; Schneider et al., 2001) (Fig. 2A). We applied conditioned media from transfected Cos7 cells, producing the recombinant ectodomain of Eda-A1 that mimicked the processed soluble endogenous ligand, to skin explants (Fig. 2B). The recombinant Eda protein runs as several bands on an SDS-PAGE gel, unlike the full-length Eda (which produces a doublet band) (Mikkola et al., 1999). It appears that these multiple bands are mainly due to differential N-glycosylation (data not shown), which appears not to affect the activity of the molecule [as we have previously shown that this recombinant molecule activates Edar in a biochemical assay (Koppinen et al., 2001)]. As a control, we used medium

either from cells transfected with Eda-A1 carrying a point mutation (Y343C) that has previously been shown to abolish receptor binding without affecting trimer formation (Schneider et al., 2001), or from cells transfected with an empty vector.

We cultured wild-type E13 skin explants, which are still devoid of hair placodes, in the presence of conditioned medium from *Eda-A1* transfected cells, and analysed the forming placodes by whole-mount *in situ* hybridisation using β -catenin as a probe. A prominent effect was detected after only 1 day of culture (Fig. 3A). As in *Eda-A1* transgenic mice, the newly formed placodes were enlarged and fusions between placodes were frequent, whereas explants cultured with conditioned medium from *Eda-A1* (Y343C) transfected or vector transfected cells appeared normal (Fig. 3B; data not shown). Apparently, Eda-A1 promoted placodal cell fate at the expense of the interfollicular fate. The effect was also evident after 2 days of culture (Fig. 3C,D). The smaller spots detected in control explants (Fig. 3D) by β -catenin-specific probe represent emerging secondary hair placodes. The fact that they are not observed in explants treated with Eda-A1 may be due to lateral inhibition by the larger placodes. If Eda-A1 was added to skin explants at E14, a time point when the primary placodes have already been formed, no effect could be seen after 24 hours (Fig. 3E,F). However, after 2 or 3 days of culture, the irregular pattern of placodes was again pronounced (Fig. 3G,H; and data not shown).

In order to control the amount of Eda protein used in the experiments, as well as to be able to apply Eda locally with beads, we also used purified recombinant Fc-Eda-A1. We used recombinant protein containing the receptor binding domain fused to the C terminus of an IgG Fc domain (Fig. 2A,C). This molecule has been shown to almost fully rescue the Eda deficiency of developing embryos when administered intravenously to pregnant *Tabby* mice (Gaide and Schneider, 2003). We first tested the ability of Fc-Eda-A1 to rescue the Eda deficiency of *Tabby* skin *in vitro*. In *Tabby* mice, the primary hair follicles fail to develop and no hair placodes are detected before E16.5-E17 (Laurikkala et al., 2002). E13 skin

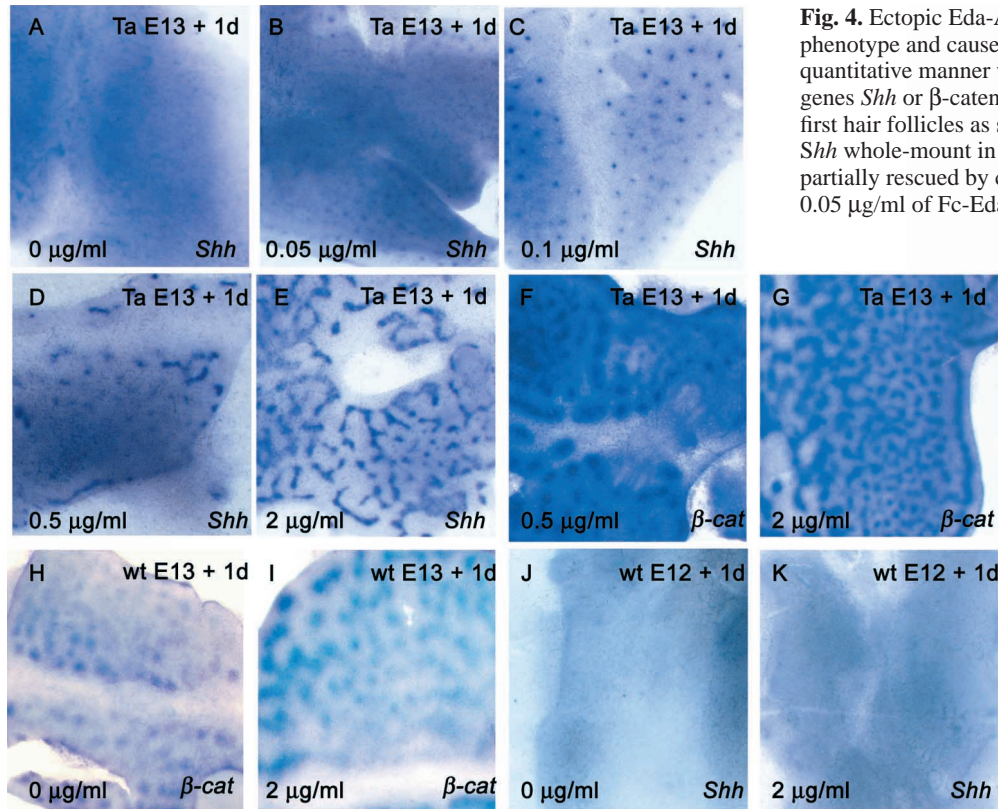


Fig. 4. Ectopic Eda-A1 protein rescued the early *Tabby* skin phenotype and caused enlargement of the placodal fields in a quantitative manner when analysed with placode marker genes *Shh* or β -catenin. Eda-deficient *Tabby* skin lacks the first hair follicles as shown by the absence of placode marker *Shh* whole-mount in situ (A). *Shh* expression in placodes was partially rescued by culturing the *Tabby* skin explants with 0.05 μ g/ml of Fc-Eda-A1 protein (B). Full rescue was obtained with 0.1 μ g/ml (C) to 0.5 μ g/ml (D) of Fc-Eda-A1. Higher concentration (2 μ g/ml) caused enlargement and fusion of the follicles as shown by *Shh* expression (E). Similarly, β -catenin expression analysis showed rescue with 0.5 μ g/ml of Fc-Eda-A1 (F) and fused follicles with 2 μ g/ml (G). Wild-type E13 skin sample cultured for 1 day showed β -catenin localisation to the forming hair placodes (H), while 2 μ g/ml concentration of Fc-Eda-A1 protein (I) caused expansion and fusion of the β -catenin expression domains of the wild-type skin. Skin explants prepared at E12 and cultured for 1 day did not show *Shh* expression (J) and no indication of placode formation was detected upon addition of 2 μ g/ml Fc-Eda-A1 to the culture medium (K).

explants were cultured in the presence of increasing amounts of recombinant protein for 1 or 2 days followed by whole-mount in situ hybridisation to analyse placode formation. After only 1 day of culture, the development of hair placodes had initiated in explants exposed to Fc-Eda-A1 but not in control explants, as evidenced by whole-mount in situ hybridisation analysis of *Shh* (Fig. 4A-E) or β -catenin expression (Fig. 4F,G). Administration of 0.05 μ g/ml of Fc-Eda-A1 already resulted in the formation of some placodes (Fig. 4A,B). With concentrations 0.1 μ g/ml (Fig. 4C) to 0.5 μ g/ml (Fig. 4D,F) of Fc-Eda-A1 we obtained rescue with regularly spaced placodes. Application of higher amounts (2.0 or 5.0 μ g/ml) resulted in a typical 'overdose' effect, i.e. enlarged and fused placodes, resulting in apparent acquisition of placodal cell fate at the expense of interfollicular cell fate, as evidenced by a stripe-like expression pattern of *Shh* (Fig. 4E,G; data not shown). Occasionally, the effect of the Fc-domain containing Eda-A1 protein was more pronounced in the outer edges of the explant (e.g. Fig. 4D), a phenomenon that was never detected with our conditioned medium containing recombinant Eda mimicking the endogenous protein. This may be due to the Fc-portion as antibodies in general have a tendency to show increased staining at the edges of skin explants. When E13 *Tabby* skin explants were cultured for 2 days, no evidence of placode formation was revealed by *Shh* probe in nontreated explants, whereas, as expected, a dose-dependent effect of Eda-A1 was apparent (data not shown).

When E13 wild-type explants were treated with purified Fc-Eda-A1, no effect was seen with low doses of Eda (data not shown). However, application of 1.0 μ g/ml or more had the same placode enlarging effect as the conditioned medium

from Eda-A1-expressing cells, and most of the cells of the explants seemed to adopt the follicular fate, verifying our previous results (Fig. 4H,I). We noticed no consistent difference in the response of wild-type and *Tabby* skin to high doses of Eda-A1. We have previously shown that in K14-*Eda-A1* mice hair follicles appear to form continuously throughout embryogenesis (Mustonen et al., 2003). This is not as evident in vitro, possibly owing to the use of higher concentrations of Eda-A1 that resulted in most of the explant adopting follicular fate. Moreover, the limited growth of the skin sample in vitro may simply result in insufficient space for new follicles to form.

Next, we tested whether we could advance the appearance of hair placodes by application of Fc-Eda-A1. Wild-type E12 skin explants were dissected and cultured with 2 μ g/ml of purified Eda-A1, and analysed for placode formation after 1 or 2 days of culture. As expected, a clear effect on placode size was detected after 2 days of culture (equivalent to E14) (data not shown). However, in line with our in vivo analysis of K14-*Eda-A1* mice, no evidence of placode formation was detected after 24 hours exposure to Eda-A1 (equivalent to E13) (Fig. 4J,K). Hence, stimulated Eda-Edar signalling did not accelerate the initiation of the first hair follicles.

Eda-A1 cannot override the inhibitory effect of Bmp4

Our results indicate that Eda-A1 promotes placodal cell fate. Exogenous Bmp4, however, has been shown to inhibit both hair and feather placode formation (Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999). Next, we wanted to test whether Bmp4 can counteract the placode promoting effect of Eda-A1. Wild-type E13 skin was cultured for 24 hours

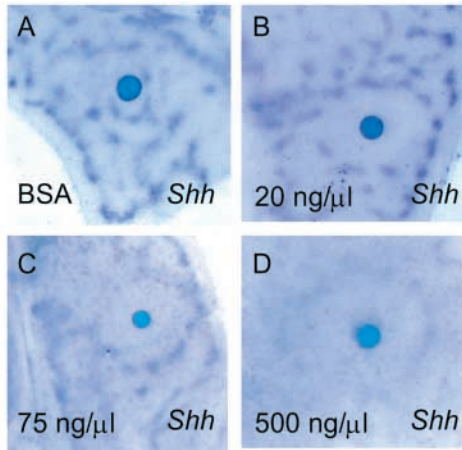


Fig. 5. Bmp4 inhibits placode formation, despite the presence of Eda-A1. E13 wild-type skin explants were grown for 24 hours in the presence of 2 $\mu\text{g}/\text{ml}$ of Eda-A1 and analysed for placode formation by whole-mount in situ hybridisation with β -catenin (data not shown) or *Shh* probe. BSA control beads (A; 0/12 explants) had no effect, whereas two agarose beads soaked in 20 ng/ μl (B; 11/12 explants), 75 ng/ μl (C; 10/10 explants) or 500 ng/ μl (D; 13/13 explants) of Bmp4 placed on top of each explant inhibited placode formation and enlargement in the vicinity of the bead.

in the presence of Eda-A1 (2 $\mu\text{g}/\mu\text{l}$) simultaneously with agarose beads releasing Bmp4 placed on top of the explants. As the effect of Bmp4 may be concentration dependent (Vainio et al., 1993), we used beads soaked in three different concentrations. The activity of Bmp4 beads was verified by their ability to induce the expression of *Msx2* (Laurikkala et al., 2002) (data not shown). At all concentrations tested, placode formation was inhibited around the Bmp4 bead (Fig. 5). With highest concentrations used, sometimes most of the explant was devoid of placodes (Fig. 5C). Whether Bmp4 specifically inhibits Eda-A1 signalling or placode initiation at an even earlier step is currently unclear.

Eda-A1 overexpression also affects molar tooth placodes

As Eda-A1 overexpression influences not only hair but also tooth and mammary gland development (Mustonen et al., 2003) we reasoned that the formation of tooth placodes might be affected. About half of the adult K14-*Eda-A1* transgenic mice have an extra tooth in front of the first molar in their lower jaws and also the shape of the molars is altered. The initiation of tooth development around E11 is marked by the appearance of a thickened stripe of the oral epithelium called dental lamina from which the tooth placodes form (Jernvall and Thesleff, 2000). We dissected E11 to E13 lower jaws of K14-*Eda-A1* transgenic embryos and their wild-type littermates and analysed the appearance of the dental lamina and molar placodes by the expression of *Pitx2* and *Shh* using whole-mount in situ hybridisation. *Pitx2* expression is one of the earliest epithelial markers specifying the odontogenic field (Mucchielli et al., 1997), and in *Pitx2*^{-/-} embryos tooth development is arrested at an early stage (Lin et al., 1999; Lu et al., 1999). No difference was observed in *Pitx2* expression between transgenic and wild-type embryos at E11 (Fig. 6A,B).

Shh shows a very specific and punctate expression during tooth development (Keränen et al., 1998) and is confined to the forming placodes. At E11, *Shh* expression nicely marked the development of the incisor placode, whereas the expression in the molar area was still rather fuzzy (data not shown). The expression patterns in transgenic and wild-type embryos were indistinguishable at this developmental stage. By contrast, at E12, the molar placode of transgenic embryos appeared wider than that of wild-type embryos (Fig. 6C,D). For statistical analysis, whole-mount images were photographed and the area of *Shh* expression was measured. These results clearly demonstrated that the transgenic molar placodes were larger than those found in wild-type embryos (Fig. 5G), and the difference was statistically significant ($P < 0.05$). No evidence of a supernumerary placode giving rise to the extra molar, which is situated anterior to the first molar in adult K14-*Eda-A1* mice, was detected at E11-E12. However, at E13, a prominent new placode, as evidenced by *Shh* expression, had emerged in transgenic embryos in front of the molar placode (Fig. 6F). These placodes were present in all embryos analysed, although extra teeth were detected in only half of the adult transgenic animals (Mustonen et al., 2003). Interestingly, a minor *Shh* positive area was also detected in the same location in the wild-type embryos at E13 (Fig. 6E). These placodes apparently regress, as wild-type mice always had the normal number, i.e. three molars. Hence, in the transgenic mice, Eda-A1 apparently stimulated the growth of already existing placodes allowing them to develop into supernumerary teeth. The finding that only half of these transgenic placodes gave rise to erupted teeth indicates that there may be a delicate balance of activating and inhibitory influences that determine whether a placode develops to a mature tooth or not.

Supernumerary placodes feature mammary gland development of K14-*Eda-A1* mice

We have previously reported that K14-*Eda-A1* transgenic females have extra nipples that are sometimes associated with small fat pads (Mustonen et al., 2003). The development of mammary glands starts at around E10.5 with the formation of two mammary (milk) lines, one line along each flank of the mouse embryo (Veltmaat et al., 2003). The first morphological sign of mammary placode formation is seen at around E11.5, when five epithelial thickenings, slightly elevated above the surface ectoderm, are seen in constant positions. *Lef1* is one of the earliest marker genes detected in mammary placodes (van Genderen et al., 1994), and the shape dynamics of *Lef1* expression, which progresses from a short line via a comet-shape to a disc-like structure, can be used to follow placode formation (Mailleux et al., 2002). Therefore we analysed the newly formed placodes of K14-*Eda-A1* embryos by whole-mount in situ hybridisation with a probe specific to *Lef1* at E11 to E13. At E11, placodes 3 and 4 had already formed and were seen as dot-like structures, whereas placode 2 still had a comet-shape appearance (Fig. 7A,B). This result is in line with previous reports revealing the asynchrony of mammary placode formation placode 2 being the last to develop (Mailleux et al., 2002). No differences between wild-type and transgenic embryos were detected at this early developmental stage. However, at E11.5 all transgenic embryos were easily distinguished from their wild-type littermates based on the appearance of additional *Lef1* expression areas along the

mammary line (Fig. 7C,D). At E12 (Fig. 7E,F) and E13 (data not shown) supernumerary placodes were detected in all transgenic embryos analysed ($n=13$) in contrast to the normal five placodes in wild-type littermates ($n=7$). All K14-*Eda-A1* embryos had either seven or eight *Lef1*-positive placodes in each mammary line (average 7.3), typically the same number on both sides of the body.

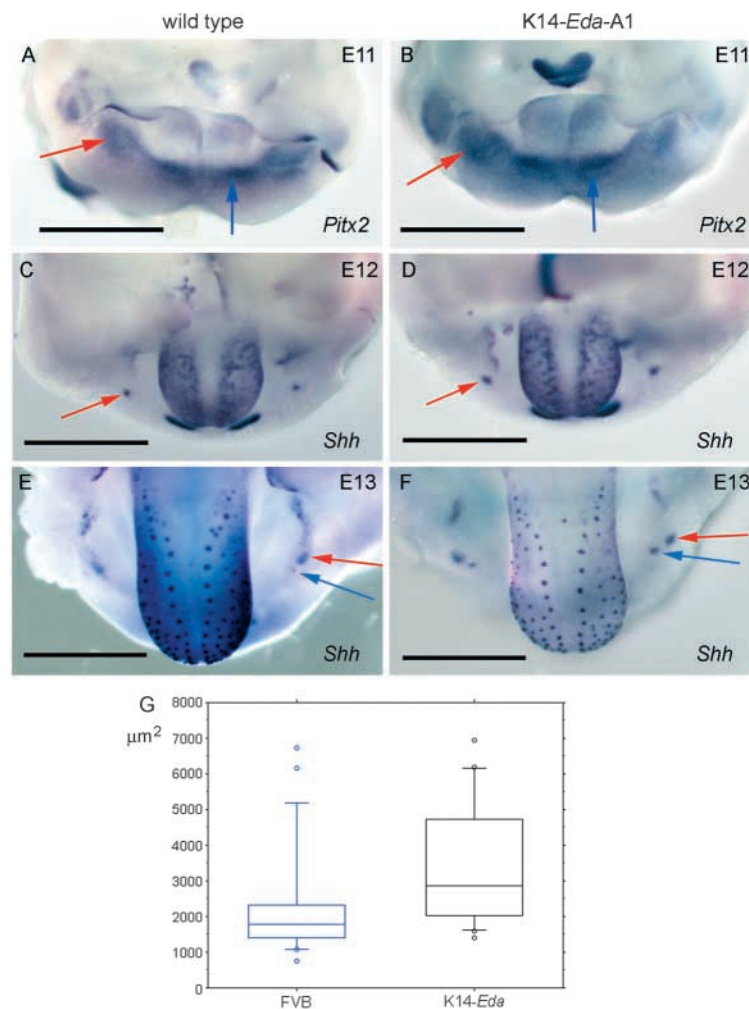


Fig. 6. Eda-A1 overexpression caused enlargement of the first molar placode at E12 and an extra molar placode in E13 lower jaws. (A-F) Top views of lower jaws. The odontogenic molar (red arrows in A and B) and incisor (blue arrows in A and B) fields were visualised in E11 embryonic lower jaws by *Pitx2* expression. No difference was seen between wild-type (A) and K14-*Eda-A1* transgenic (B) embryos. At E12, the dental placodes were visualised by *Shh* expression (C,D). The molar placodes were larger in transgenic embryos when compared with controls (arrows in C,D). At E13, extra placodes (blue arrow) were located in front of the transgenic molar placodes (red arrow in F). However, a small *Shh*-positive placode was also detected in wild-type embryos (blue arrow in E). The size measurements of E12 mandibular molar placodes of K14-*Eda-A1* and control mice are presented as a box plot presentation (G). The size measure is a product of width and length of a placode. The height of the box presents 50% of the results. Mean is shown as a horizontal line inside the box and 95% confidence limits are shown by the lines outside the box. The difference between groups is statistically significant ($P=0.03$) according to the Mann-Whitney U-test. Measurements were made on three litters of mice. Number of placodes measured: K14-*Eda-A1* $n=23$; wild-type littermates, $n=18$. Scale bars: 1 mm.

The position of the normal five placodes was not altered in transgenic embryos. The extra placodes were situated in all cases between the third (thoracic) and the fourth (inguinal) placodes, a region that is characterised by a long inter-placode distance. Analysis of adult female transgenic mice ($n=16$) revealed on average 6.9 nipples per milk line, suggesting that most, but possibly not all, supernumerary mammary gland placodes progress beyond early stages and persist until adulthood. The effect of *Eda-A1* on mammary placodes hence resembled its effect on dental placodes. In both cases, the extra placodes formed close to existing placodes and along the predetermined ectodermal stripes, the mammary line and dental lamina.

The increased hair placode size of K14-*Eda-A1* embryos is not due to proliferation

Basically, the growth of an epithelial thickening or placode could result from local cell proliferation, cell migration, altered cell adhesion or all of them. To learn more about the mechanistic role of *Edar* signalling in placode formation, we examined cell proliferation by counting mitotic indexes of newly formed hair placodes at E14. Pregnant wild-type mice mated with K14-*Eda-A1* males were injected with BrdU and sacrificed 2 hours later. Epithelial cells of skin sections from equivalent back-skin regions were analysed for BrdU incorporation by immunohistochemical staining (Fig. 8). First, we counted mitotic indexes (percentage of BrdU-positive cells) from wild-type hair placode and interplacode regions (Table 1). Proliferation was detected in both regions. However, the interplacodal area showed much higher mitotic index (38.6%) than the placode area (24.4%) ($P=0.004$). Similar figures were obtained from analysis of transgenic embryos: 42.9% of interplacode cells were BrdU positive compared with 24.2% of placode cells ($P<0.001$). No differences in mitotic figures were observed between wild-type and transgenic animals in placode area (24.4% versus 24.2%, $P=0.964$), or in interplacode region (42.9% versus 38.6%, $P=0.338$). This suggests that although increased *Edar* signalling in the transgenics results in larger placodes, this phenomenon was not due to propagated proliferation.

Discussion

Epithelial placodes are examples of cell aggregates that initiate the development of most ectodermal organs. It is typical for ectodermal organs that their placodes develop sequentially in specific patterns. For example, feathers form in strict hexagonal patterns, whereas teeth and mammary placodes form sequentially at precise locations along ectodermal stripes, the dental lamina and mammary line, respectively. It is apparent that in all cases individual primordia need signals for their initiation, expansion and termination. The molecular signals regulating the spacing and growth of hair and feather placodes have been elucidated to some extent, and it is thought that

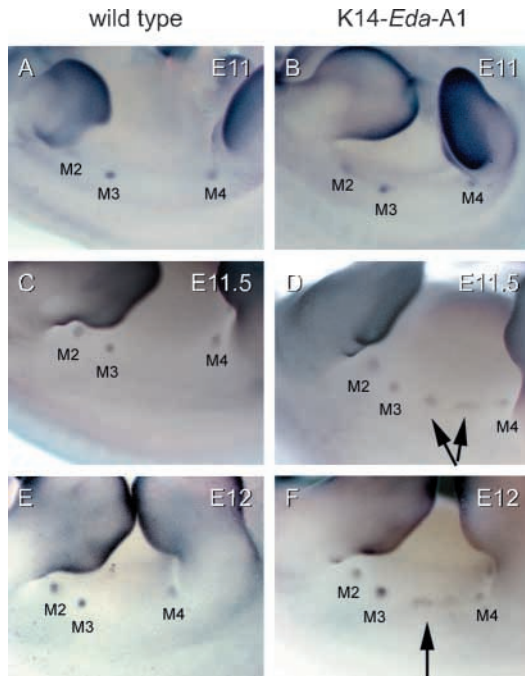


Fig. 7. K14-*Eda-A1* transgenic mice had ectopic mammary placodes, as visualised by whole-mount in situ hybridisation with *Lef1* probe. At E11 the early placodes were initiated in both the wild-type (A) and transgenic (B) embryos. Mammary placodes 3 and 4 were seen as clear dots (M3, M4). Placode 2 (M2) had still a comet shaped appearance at this developmental stage. Placodes 1 and 5 were hidden by the developing limbs. At E11.5, M2-M4 are seen as clear dots in wild-type embryos (C), whereas supernumerary placodes (arrows) between M3 and M4 are evident in transgenic animals (D). At E12, the extra placodes were still visible in the transgenics (arrow in F) in contrast to the normal five placodes of wild-type animals (E).

both activators and inhibitors of follicle fate are involved. A model has been put forward that proposes that a primary inductive signal triggering the whole cascade initially occurs uniformly throughout the skin. It activates both promoters and repressors of placodal fate, and their random intrinsic instability (combined with their local competition with one another) then restricts the size and site of the placodes, resulting in the establishment of a periodic pattern (Turing, 1952; Koch and Meinhardt, 1994; Jung and Chuong, 1998; Barsh, 1999; Jiang et al., 1999). In line with this model, several positive and negative regulators of follicle fate are initially expressed uniformly in the skin and become localised upon placode formation (Millar, 2002). The colocalisation of the

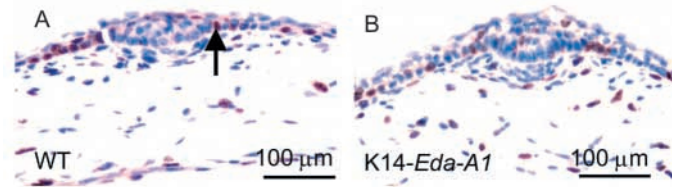


Fig. 8. Overexpression of *Eda-A1* in embryonic ectoderm did not increase proliferation in hair placodes or developing skin. The amount of BrdU positive S-phase proliferating cells (arrow) in E14 hair placodes was smaller than in the interplacodal areas in both the wild-type (A) and transgenic skin (B) sections.

activators and the inhibitors within the placode (as opposed to the inhibitors being localised to the interplacode region) favours the above described reaction-diffusion model (Turing, 1952), which assumes that within the placode the effective concentration of the activators of placode fate is higher than that of the inhibitors whereas the latter ones diffuse further than the activators, thus defining the borders of the placodes. The effect of repressors within the primordium may also be prevented by their specific inhibitors (Botchkarev et al., 1999).

Our results from the analysis of *Eda-A1*-overexpressing mouse embryos indicate that increased signalling via *Eda-A1* receptor *Edar* promotes placodal fate in developing mammary glands and teeth, as well as in the first wave of hair follicles that give rise to guard hairs. In particular, the placodes of molar teeth as well as guard hairs were increased in size. The more thorough analysis of the effects *Eda-A1* recombinant protein on hair placodes in organ cultures indicated that the stimulation of placodal fate took place at the expense of interplacode region, as evidenced by the expression patterns of placodal marker genes as well as histology. Clearly, lateral inhibition regulating the size as well as the distance of placodes from one another was relieved. In principle, this could be achieved either by inhibiting the action of molecules that mediate lateral inhibition or by promoting the action of placodal activators. This may also account for the other phenomena typical to K14-*Eda-A1* mice we have reported earlier, i.e. the continuous development of new hair follicles abnormally close to the pre-existing ones, and the development of extra teeth and mammary glands (Mustonen et al., 2003). Recently, several other reports on transgenic mice overexpressing *Eda-A1* have been published (Srivastava et al., 2001; Cui et al., 2003; Zhang et al., 2003; Newton et al., 2004). None of these reported any changes in mammary glands or teeth, whereas defects in distinct hair types were frequent. Cui et al. used a full-length *Eda-A1* and a Tet-inducible promoter, whereas Newton et al. expressed the ectodomain of *Eda-A1* under K5 or muscle

Table 1. Mitotic indices of wild-type and K14-*Eda-A1* E14 skin

	Interfollicular area	Hair follicle area	<i>P</i>
Wild type	38.6 (<i>n</i> =3441 cells)	24.4 (<i>n</i> =26 follicles; <i>n</i> =818 cells)	0.004
K14- <i>Eda-A1</i>	42.9 (<i>n</i> =2584 cells)	24.2 (<i>n</i> =40 follicles; <i>n</i> =1937 cells)	<0.001
<i>P</i>	0.338	0.964	

Mitotic indices, i.e. the proportions (%) of S-phase BrdU positive cells in the E14 back skin sections were smaller in the hair follicle areas than in the interfollicular epithelium in both wild-type and K14-*Eda-A1* transgenic skin. The difference between the follicular and interfollicular mitotic indices was significant in both wild-type (*P*=0.004) and transgenic (*P*<0.001) skin sections. The differences of the mitotic indices between wild-type and transgenic interfollicular epithelia were not significant (*P*=0.338). In addition, the follicular mitotic indices were similar between the transgenic and wild-type skin sections (*P*=0.964).

specific myosin light-chain 2 promoter. The two groups reported opposite results on the effect of Eda-A1 on hair follicle density in adult mice. This, in principle, could be taken as an indication of the role of Eda-A1 in the regulation of follicle fate. Unfortunately, no embryonic analyses were made. Zhang et al. used the involucrin promoter to drive the expression from E16.5 onwards, and thus no effects on primary hair placodes could be expected. At later stages, they found, at periodic locations, multiple hair follicles side by side, as well as branched follicles, similar to previous reports (Mustonen et al., 2003). In line with our results, occasionally hair buds with increased width were detected, suggesting that Eda-A1 also affects the expansion of the secondary placodes.

In the present study, we demonstrated that the placodes for the extra teeth and mammary glands developed in close proximity to the pre-existing placodes on the dental lamina and mammary line. During feather and hair development, Bmps of the transforming growth factor β (TGF β) family are the best characterised candidates for placode inhibitors and mediators of lateral inhibition, whereas during tooth and mammary gland development lateral inhibition has been less studied. Ectopic expression of Bmp2 or Bmp4, or beads releasing Bmp4 inhibit hair and feather placode formation (Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999), and our data showed that the placode promoting effect of Eda-A1 was inhibited by Bmp4. Forced expression of the Bmp inhibitor noggin during early feather development results in fused and enlarged placodes (Jung et al., 1998; Noramly and Morgan, 1998). Noggin also promotes murine hair placode formation (Botchkarev et al., 1999). Hence, these phenotypes are very similar to the effects of increased Eda-A1 signalling we observed both in vivo and in vitro.

Molecules that promote placode fate include members of the Wnt and Fgf pathway (Pispa and Thesleff, 2003). Exogenous Fgfs induce ectopic placodes in wild-type chick embryos (Song et al., 1996; Jung et al., 1998). In mice, expression of a soluble dominant-negative form of Fgfr2 IIIb results in total absence of hair follicles in the most severely affected embryos (Celli et al., 1998). Fgf signalling is also indispensable for tooth and mammary gland development (Trumpp et al., 1999; Mandler and Neubüser, 2001; Maillieux et al., 2002). Canonical Wnt activity is mediated by the co-operation of β -catenin with transcription factors of the Lef1/TCF family. Interestingly, forced stabilisation and activation of β -catenin in developing chick skin can override lateral inhibition and elicit a placodal response in interfollicular ectoderm (Noramly et al., 1999). This very much resembles the response of mouse skin to excess of Eda-A1. Constitutively active β -catenin can also induce ectopic hairs (Gat et al., 1998), whereas lack of β -catenin in the developing ectoderm inhibits hair follicle initiation (Huelsenken et al., 2001). In conclusion, the effect of Eda-A1 is highly similar to the best characterised proteins promoting hair follicle fate.

What is then the position of Edar signalling in the hierarchy of molecules regulating placodal cell fate? It is well established that the primary inductive signal at least during hair and mammary gland development is derived from the mesenchyme, whereas both Eda and Edar are expressed by the ectoderm (Laurikkala et al., 2001; Laurikkala et al., 2002). We were not able to accelerate the initiation of hair follicles either in vivo or in vitro by exogenous Eda-A1, suggesting that Eda-

A1 cannot override the normal requirement of the first dermal signal. Accordingly, the initial development of the mammary line and five mammary primordia were normal in K14-*Eda-A1* mice. In addition, the dental lamina, the incisor and molar placodes were normally defined in time and space. Ectopic mammary and tooth placodes were detected slightly later and only next to already formed placodes within the already specified morphogenetic fields, mammary line and dental lamina, respectively. All these data combined with earlier results (see Introduction) suggest that Eda-A1 does not regulate the primary induction of epithelial placodes, but instead plays a pivotal role in the next phase, i.e. in the expansion of the placodes. In this process, Eda-A1 signalling apparently is very high in hierarchy. The fact that in the absence of Eda activity only some placodes are totally missing (primary and some of the secondary hair placodes but not tooth and mammary gland primordia) may merely reflect redundancy with another TNF family member or with a parallel signalling route required for the same process. Intriguingly, TNFRSF19, an orphan TNF receptor with sequence similarity to Edar shows close co-expression with Edar in tooth, hair and mammary placodes (Pispa et al., 2003).

The cellular mechanism regulating epithelial placode formation has been a neglected area of research, despite being a matter of dispute for decades (Magerl et al., 2001; Pispa and Thesleff, 2003; Veltmaat et al., 2003). Our results have two main implications. First, hair placode formation seems not to result from a locally enhanced proliferation of placodal cells. Almost twice as much proliferation was detected in the interplacode area as compared with the placode area. These results are in line with data from Wessells and Roessner (Wessells and Roessner, 1965) who studied ³H-thymidine incorporation and mitotic figures of hair follicle cells. They found that primary hair and vibrissae placodes were almost devoid of label, and the incorporation of label continued only after the placode had invaginated in the mesenchyme. Similar results were obtained during feather development (Wessells, 1965). However, the contrary has been suggested based on the fact the hair placodes in newborn mice (giving rise to auhene and zigzag hairs) are positive for the proliferation marker Ki67 (Magerl et al., 2001). During mammary gland development, the hypothesis of cell migration being the driving force underlying the formation of mammary placodes has got more support (Veltmaat et al., 2003). As in hair and feather placodes, the mammary region has lower mitotic index than the adjacent ectoderm (Balinsky, 1950). In addition, the shape of the epithelial cells of the mammary line (Propper, 1978) as well as the dynamic expression profiles of mammary placode marker genes (Maillieux et al., 2002) have been interpreted as strong evidence to support cell migration as the primary mechanism of placode formation.

The second implication of our BrdU incorporation data is that the specific role of Edar signalling during the growth of the placode appears not to involve cell proliferation. The mitotic indices of wild-type and K14-*Eda-A1* epidermis were highly similar, yet the placodes of transgenic animals were larger, indicating that Eda-A1 regulates their growth via another mechanism. In addition, we have not been able to detect enhanced proliferation in skin explants using Eda-A1-releasing beads (T.M. and M.L.M., unpublished). Moreover, Eda promoted not only hair but also tooth and mammary

placode fate, suggesting that it has a similar role during the early stages of the development of most ectodermal appendages. The next task is now to reveal what cellular processes Eda-A1-Edar signalling controls. We find it unlikely that Eda-A1 suppresses apoptosis, as the epidermis of *Tabby* embryos does not show increased cell death (J.L. and I.T., unpublished) nor does the enamel knot, the site of *Edar* expression of the developing molar (Koppinen et al., 2001). Regulation of cell-cell contacts is intimately involved in hair follicle formation and downregulation of the intercellular adhesion molecule E-cadherin appears to be essential for hair placode formation (Jamora et al., 2003). This is achieved through the concerted action of a Bmp inhibition, which is required for the expression of Lef1, and of a Wnt molecule, which is required for the stabilisation of β -catenin to make a functional Lef1/ β -catenin complex that directly binds to E-cadherin promoter. Eda-A1 could be involved in the control of cell adhesion either directly or indirectly. However, it could promote the migration of epithelial cells towards the placode and by this means regulate the growth of the placode. Of course, the effects of Eda-A1 need not be direct and could be mediated via other known placode-promoting signals. The downstream responses of Edar are probably mediated by transcription factor NF- κ B as Edar signalling activates the NF- κ B pathway in cultured cells (Yan et al., 2000; Koppinen et al., 2001; Kumar et al., 2001), and mutations in molecules required for the NF- κ B activation result in HED in humans (Zonana et al., 2000; Smahi et al., 2002; Courtois et al., 2003). However, the target genes of Edar signalling are still unknown.

In conclusion, we have shown that Eda-A1-Edar signalling stimulates the formation of ectodermal placodes in hair, teeth and mammary glands. However, Eda-A1 did not affect the initiation of the first forming placodes in any of the organ systems, indicating that it apparently does not influence the initial patterning of the organs. It is plausible that the defects in the patterning and morphogenesis of the ectodermal organs in humans and mice with loss of function of Eda-Edar signalling as well as in the transgenic mice overexpressing Eda-A1 result from early effects on ectodermal placode formation. The significance of an early function of Eda-A1 is supported by recent experiments where the *Tabby* phenotype was rescued by providing recombinant Eda-A1 into the uteri of pregnant mice (Gaide and Schneider, 2003). Finally, our observations on the effects of increased Eda-A1 function both in vivo and in vitro indicate that it stimulates placode enlargement by a mechanism not involving cell proliferation. It apparently affects the balance of activators and inhibitors, and impairs the lateral inhibition mechanism responsible for both the expansion of the placodes and initiation of successive placodes.

We thank Riikka Santalahti, Merja Mäkinen, Heidi Kettunen and Marina Lotopena for skillful technical help. The work was supported by grants from the Finnish Academy, Emil Aaltonen Foundation and Sigrid Jusélius Foundation.

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