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Stimulation of ectodermal organ development by Ectodysplasin-A1

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Abstract

Organs developing as ectodermal appendages share similar early morphogenesis and molecular mechanisms. Ectodysplasin, a signaling molecule belonging to the tumor necrosis factor family, and its receptor Edar are required for normal development of several ectodermal organs in humans and mice. We have overexpressed two splice forms of ectodysplasin, *Eda-A1* and *Eda-A2,* binding to Edar and another TNF receptor, Xedar, respectively, under the keratin 14 (K14) promoter in the ectoderm of transgenic mice. *Eda-A2* overexpression did not cause a detectable phenotype. On the contrary, overexpression of *Eda-A1* resulted in alterations in a variety of ectodermal organs, most notably in extra organs. Hair development was initiated continuously from E14 until birth, and in addition, the transgenic mice had supernumerary teeth and mammary glands, phenotypes not reported previously in transgenic mice. Also, hair composition and structure was abnormal, and the cycling of hairs was altered so that the growth phase (anagen) was prolonged. Both hairs and nails grew longer than normal. Molar teeth were of abnormal shape, and enamel formation was severely disturbed in incisors. Furthermore, sweat gland function was stimulated and sebaceous glands were enlarged. We conclude that ectodysplasin–Edar signaling has several roles in ectodermal organ development controlling their initiation, as well as morphogenesis and differentiation.
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Keywords: Tabby; *downless*; Edar; Eda; Hair development; Tooth development; Mammary gland development

Introduction

A variety of organs in vertebrates develop as appendages of embryonic ectoderm. These include hairs, feathers, scales, nails, teeth, and many exocrine glands such as sweat glands and mammary glands. Interactions between the ectoderm and underlying mesenchyme regulate their morphogenesis, and the developmental regulatory molecules are also generally shared between the different organs (Thesleff and Mikkola, 2002b; Millar, 2002). The initiation of the organs developing as ectodermal appendages is marked by epithelial thickenings, or placodes. A characteristic feature of placodes is that they express many signal molecules, including sonic hedgehog (SHH) and several bone morphogenetic proteins (BMPs), Wnts, and fibroblast growth factors (FGFs), and they therefore can be defined as embryonic

signaling centers. Hair development is initiated by an early dermal message inducing placode formation in ectoderm. Placodal signals then regulate dermal cell condensation and dermal papilla development, and the subsequent dermal messages regulate the growth of the ectodermal placode and its invagination (Millar, 2002). In teeth, an early signal from epithelium induces dental competence in the underlying, neural crest-derived mesenchyme which signals back to ectoderm inducing placode formation and growth of the tooth bud. Epithelial–mesenchymal interactions later instruct also the morphogenesis of the tooth crown and differentiation of the cells producing the dental hard tissues. Mammary glands form from two ectodermal thickenings called milk lines on the sides of the early embryo via similar morphological stages involving reciprocal signaling between the epithelium and the mesenchyme. Later development leads to the formation of the nipple and the branching morphogenesis of premature mammary glands, which mature only after hormonal stimuli during puberty, pregnancy, and birth of the pups (Hennighausen and Robinson, 2001).

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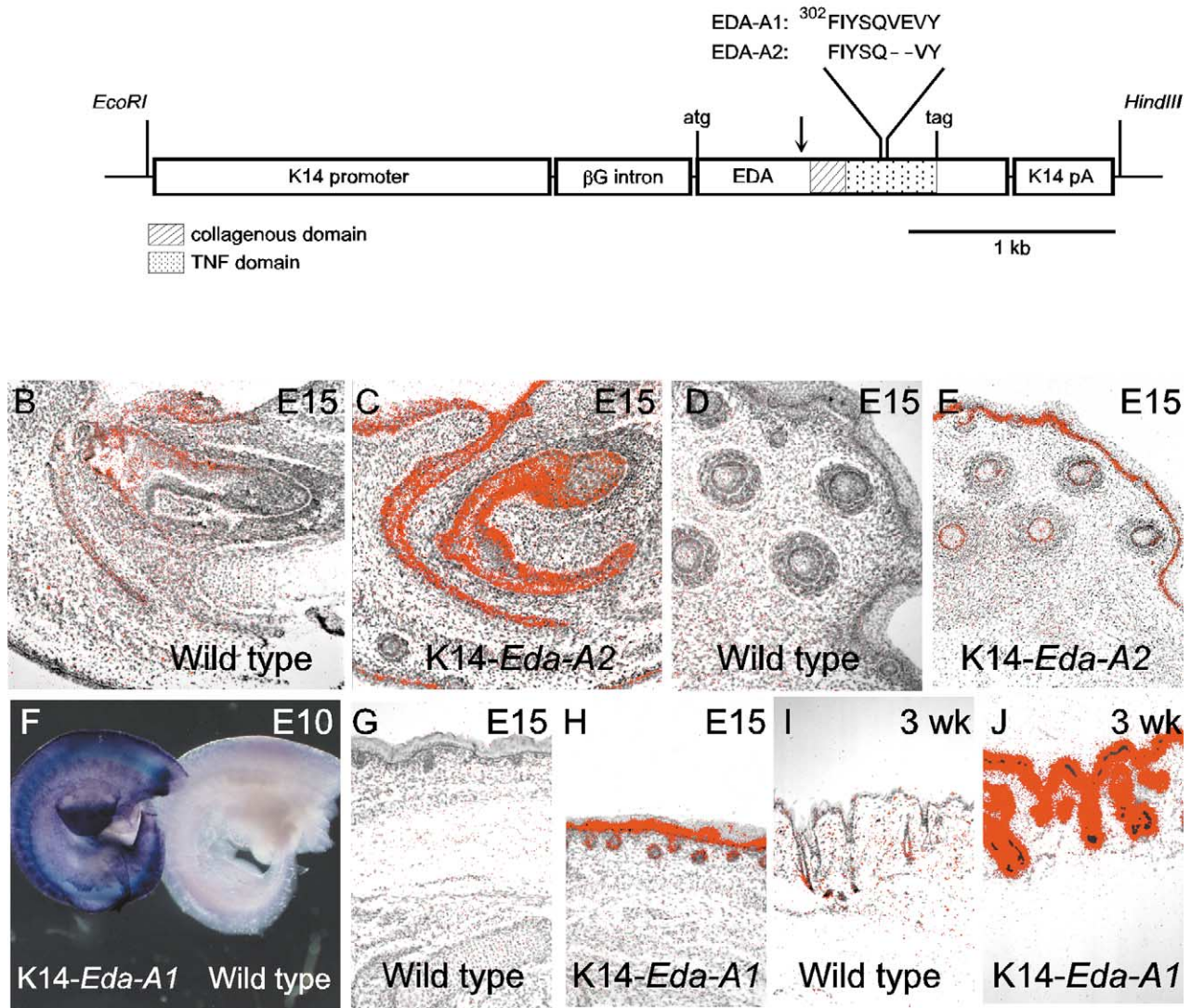*E-mail address:* irma.thesleff@helsinki.fi (I. Thesleff).

Fig. 1. Keratin 14 promoter drives ectopic *Eda-A1* and *Eda-A2* expression. (A) K14-*Eda-A1* and K14-*Eda-A2* expression constructs. The start and stop codons of *Eda* cDNA are indicated as well as the difference between Eda-A1 and Eda-A2 protein sequences. The arrow indicates the furin cleavage site required for the release of a biologically active soluble protein, which consists of the collagenous domain followed by the TNF domain. K14, keratin 14; βG, β-globin; pA, polyadenylation signal. (B–E) Ectopic expression of *Eda-A2* is evident at E15 in the dental and oral epithelium of the incisor (C) and in whisker follicles and the basal layer of the skin epithelium (E) of a K14-*Eda-A2* embryo. (F–J) K14-*Eda-A1* embryos express *Eda* at a much higher level than wild type embryos at E10 (F). At E15, the ectopic expression is seen in hair follicles and the basal epithelial cell layer (G, H). Strong expression continues in 3-week postnatal skin (I, J). In situ hybridization exposure and staining times were short in order to better visualize the ectopic *Eda* expression and to avoid overexposure. Therefore, the level of wild type *Eda* expression seen here (B, D, F, G, I) does not correspond exactly to the level of endogeneous *Eda* expression.

Hypohidrotic ectodermal dysplasias (HEDs) are malformation syndromes in humans and mice characterized by severe defects in hair formation, missing and abnormally shaped teeth, and inhibited development and function of sweat glands as well as other exocrine glands (OMIM; Falconer et al., 1951; Sofaer, 1969). Ectodysplasin (Eda), which belongs to the tumor necrosis factor (TNF) family of signaling molecules, as well as its receptor Edar and the Edar-binding death domain adapter protein Edaradd were discovered through cloning of genes mutated in these syndromes and in their corresponding mouse mutants (called

Tabby, downless, and *crinkled,* respectively) (Thesleff and Mikkola, 2002a). Similar to many other TNFR family members, downstream responses of Edar are most likely mediated by NF-κB (Yan et al., 2000; Kumar et al., 2001; Koppinen et al., 2001).

Two functional isoforms of ectodysplasin, Eda-A1 and Eda-A2, have been described which differ only by two amino acids due to the usage of alternative splice sites (Srivastava et al., 1997; Bayés et al., 1998; Mikkola et al., 1999; Fig. 1A). Surprisingly, only Eda-A1 was shown to bind Edar, whereas Eda-A2 is a specific ligand for Xedar,
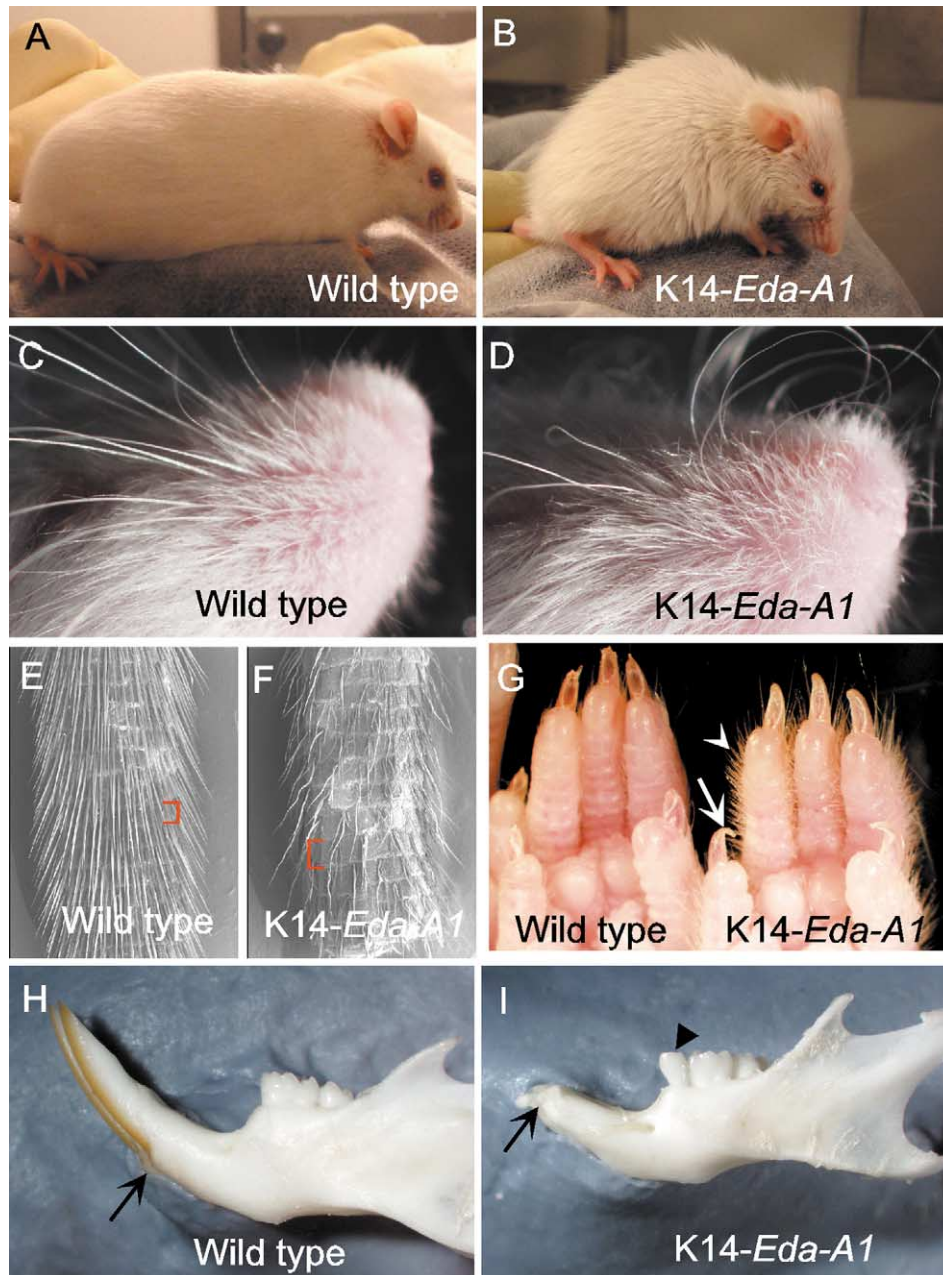
Fig. 2. *Eda-A1* overexpression affects the development of several ectodermal organs. (A, B) K14-*Eda-A1* mice show a shaggy appearance. (C–F) Wild type mice have straight whiskers (C) and tail hairs (E), but the whiskers (D) and tail hairs (F) of two K14-*Eda-A1* transgenic lines are curly. The scale-like folds of tail skin are larger in all transgenic lines as shown by SEM (brackets in E and F). (G) Nails (arrow) and hairs (arrowhead) are longer in the feet of the transgenic animals as compared with wild type. (H, I) K14-*Eda-A1* lower incisors wear down excessively (arrows in H and I indicate the bone margin). In addition, an extra molar is often seen in the transgenic lower jaws (arrowhead).

another related but distinct TNF receptor (Yan et al., 2000). However, the phenotypes of *Tabby* (null for both isoforms), *downless,* and *crinkled* mice are identical (Blake et al., 2002). As no mouse mutants nor human syndromes with Xedar mutations have been described, the relative importance of the two splice variants has remained unclear. Recently, it was shown that Eda-A1 isoform can almost completely rescue the hair and sweat gland phenotype of *Tabby* mice, suggesting that the Xedar pathway has a more subtle function during organogenesis (Srivastava et al., 2001).

Wild type mice have four different types of hair which develop in successive waves starting at embryonic day 14 (E14) (Mann, 1962). *Tabby/downless/crinkled* mice have only one hair type, abnormal hairs resembling awls. *Tabby* mice lack the first wave of hair follicles, giving rise to the long guard hairs and the last wave of follicles developing to zigzag and auchene hairs (Laurikkala et al., 2002). Thus, ectodysplasin signaling is indispensable for the formation of the first hair placodes. The expression patterns of *ectodysplasin* and *Edar* have been analyzed in detail by in situ hybridization, which, of

course, cannot distinguish between the two splice variants of *Eda*. *Edar* and *ectodysplasin* are coexpressed in the simple ectodermal sheet before the initiation of hair and tooth development is morphologically evident (Laurikkala et al., 2001, 2002). *Edar* then becomes restricted to the placodes and is downregulated in the interfollicular epidermis as well as in the other cells of oral epithelium, whereas *ectodysplasin* becomes downregulated in the placodes and shows complementary expression in the flanking ectoderm. In *Tabby* mice, *Edar* expression does not get restricted into the placodes, indicating that ectodysplasin signaling is required for the correct patterning of *Edar* expression (Laurikkala et al., 2002). Later in development, *Edar* is expressed in the secondary enamel knots which guide cusp development in molar teeth, and both *Eda* and *Edar* are expressed in the hair bulbs at the time of hair shaft formation. Thus, Edar and ectodysplasin mediate signaling within the ectoderm and not between the ectoderm and mesenchyme. However, their signaling function associates with epithelial–mesenchymal interactions during all stages of development and is integrated with other signaling pathways regulating hair and tooth development. Ectodysplasin expression is induced by Wnt signals, whereas the TGFβ signal activinβA, which is expressed in the mesenchyme beneath both hair and tooth placodes stimulates Edar expression in the placodes (Laurikkala et al., 2001, 2002). Edar appears to be upstream of most of the signal molecules expressed in the hair placodes (Huelsken et al., 2001; Laurikkala et al., 2002). However, it is not known at present whether any of these signals are directly regulated by ectodysplasin.

In order to further investigate the role of ectodysplasin, we overexpressed *Eda-A1* and *Eda-A2* splice forms under the keratin 14 (K14) promoter in the developing ectoderm of transgenic mice. *Eda-A2* overexpression did not cause any detectable phenotype. On the contrary, overexpression of *Eda-A1* resulted in alterations in a variety of ectodermal organs, including hairs, teeth, and mammary, sweat, and sebaceous glands. Extra teeth were formed, and supernumerary mammary glands with abnormal nipples were found in transgenic adult females. The morphology of molar tooth crowns was altered, and the enamel of incisors was severely disturbed. Hair composition of adult mice was abnormal, and instead of normal waves of hair follicle development, hair development was initiated continuously from E14 until birth. We suggest that Eda-A1 signaling has several roles in ectodermal organs controlling their initiation, morphogenesis, as well as differentiation.

Materials and methods

Generation of K14-Eda-A1 and K14-Eda-A2 mice

The cDNAs for mouse ectodysplasin A1 and A2 isoforms were excised with *Nco*I and *Bam*HI from the corresponding cDNAs (Srivastava et al. 1997; Mikkola et al., 1999). The blunt-ended fragment was cloned into the

*Bam*HI site of K14-BG (Gat et al., 1998), producing the K14-BG-TA1/1 and -TA2 plasmid. The transgene was released from the vector by digestion with *Eco*RI and *Hin*dIII and microinjected into the pronuclei of fertilized eggs of FVB/N mice (Jackson Laboratories, Bar Harbor, ME). Four K14-*Eda-A2* and 12 K14-*Eda-A1* DNA-positive founder mice were identified by PCR using a forward primer annealing to K14 sequence 5′-ACATCCTGGTCATCATC-CTGCC-3′ and the reverse primer 5′-GATCTTCTCCCG-GTTCCAAAG-3′ complementary to Eda sequence. The result was verified by Southern blotting. Five K14-*Eda-A1* and 4 K14-*Eda-A2* founder animals were further bred to the FVB/N strain to generate positive lines. Because of the obvious enamel defects of the *Eda-A1* overexpressing mice, the upper incisors of the transgenic animals older than 5 weeks were cut once in 2 weeks for the welfare of the animals, and the animals were provided with soft diet. Two female K14-*Eda-A1*-expressing founders were provided nutritional supplement (Nutriplus gel, Virbac) when pregnant and nursing, and their sons were used for further breeding. The generation of the transgenic K14-Eda animals had a full permission from the committee for the experimental animals of University of Helsinki, with a license number STU513A.

Hair analysis

Hairs from four individual 6- to 11-week-old wild type FVB/N and four littermate transgenic K14-*Eda* mice were plucked from the upper back skin and examined under a stereomicroscope. Back skin, tail, and whisker hairs were photographed, and all pelage hair types from back skin were measured.

Sweat analysis

Feet of the adult sedated normal and transgenic mice were cleaned and air dried. Iodine solution (2% in ethanol) was applied on the feet and air dried for 5 min. Starch–castor oil mixture (1 g/ml) was applied on the feet, and the appearing sweat glands were photographed.

Tooth analysis

Skeletal preparations of skulls of the mice were made by boiling in tap water for 30 min. Samples were then cleaned under preparation microscope and whitened by 6% hydrogen peroxide for 15 min. Jaws were photographed coronally to study the molar form. Sagittal photographs were taken for comparison of the tooth wear.

Mammary gland staining

Female virgin, pregnant, and nursing adult mice were sacrificed, and mammary gland fat pads were dissected.

Hematoxylin staining of whole-mount mammary glands was performed according to Brantley et al. (2001).

Histology and in situ hybridization

Tissues from mouse embryos were dissected in PBS under a stereomicroscope. These as well as tissues from adult mice were fixed in 4% paraformaldehyde over 2 nights, dehydrated, embedded in paraffin, and serially sectioned. Mineralized dental tissues were decalcified before paraffin embedding by using 2.5% paraformaldehyde and 12.5% EDTA in PBS. For light microscopy, the sections were stained by hematoxylin–eosin using standard protocols. The structure of tooth enamel was analyzed by using metylmetachrylate-embedded ground sections. For plastic embedding, the mandibles were first fixed in 4% paraformaldehyde for 5 days, dehydrated in an alcohol series, and kept in metylmetachrylate for 4 days before adding benzyl peroxidase catalysator (20 mg/ml). Tissues were then moved in glass vials to 37°C waterbath to solidify. Ground sections were serially sectioned at 100 μm, dried under pressure, mounted unstained with coverslides, and photographed.

Radioactive in situ hybridization on paraffin sections was performed as described in Wilkinson and Green (1990). For ^{35}S-UTP-labeled riboprobes, the following plasmids were used as templates: *Eda* and *Edar* (Laurikkala et al., 2001), *Msx1* (Vainio et al. 1993). Whole-mount in situ was performed by InsituPro robot (Intavis, Germany), and the protocol was as described in Kettunen and Thesleff (1998). Digoxigenin-labeled antisense riboprobe was synthesized for Eda mRNA (Laurikkala et al., 2001).

Scanning electron microscopy (SEM)

Adult mouse tail samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for at least 2 h, dehydrated in a graded series of 50, 70, 94, and 100% ethanol for 30 min each, and subjected to critical point drying (Bal-Tech CPD 030, Balzers, Liechtenstein). Subsequently, the samples were coated with platinum (Agar Sputter Coater, Agar Scientific, Stansted, UK) and viewed with Zeiss DSM 962 scanning electron microscope (Zeiss, Oberkochen, Germany).

Results

Overexpression of the Eda-A2 splice form had no detectable effects

Ectodysplasin has two functional splice variants, Eda-A1 and Eda-A2, which bind to and activate two different receptors, Edar and Xedar, respectively (Yan et al., 2000). Edar has been shown to be important for ectodermal organ development, while the role of Xedar has remained unclear.

To study the functional differences of Eda-A1 and Eda-A2 in ectodermal organ development, we expressed both splice forms under the keratin 14 (K14) promoter in transgenic mice (Fig. 1A). K14 promoter drives the ectopic expression to the developing ectoderm already at embryonic day 9 (E9) and later to the basal layer of the skin, the outer root sheath of the hair follicle (Vassar et al., 1989; Byrne et al., 1994), as well as the dental and oral epithelia (Dassule et al., 2000).

The four transgenic mouse lines overexpressing *Eda-A2* under K14 promoter showed no obvious phenotype. Expression of the transgene was confirmed by radioactive in situ hybridization (Fig. 1B–E). The hair types of the pelage, the tooth shape, as well as histology of developing hair follicles and tooth germs in embryos were analyzed but no abnormalities were detected (data not shown).

Overexpression of Eda-A1 affected the development of several ectodermal organs

Out of 12 transgenic *Eda-A1* overexpressing mice, 10 showed a clear and consistent phenotype, and 5 well breeding mice were chosen for generating lines for detailed analysis of the phenotype. *Eda* is normally expressed in the developing skin epithelium already at E11 (Laurikkala et al., 2002). At E14, the expression is downregulated at developing placodes but continues in the interfollicular epidermis, and later, the expression is intense in the bulb of the hair follicles. Expression of K14-*Eda-A1*-transgene driven mRNA was verified by whole-mount in situ hybridization at E10 (Fig. 1F) and by radioactive in situ hybridization at E15 and 3 weeks postnatal skin (Fig. 1G–J). The ectopic *Eda-A1* expression was markedly stronger than the endogenous expression in the ectoderm throughout the embryo. Later, the ectopic expression was seen in the basal layer of the skin, outer root sheath of the hair follicles, olfactory epithelium, neuroepithelium, and in the oral and dental epithelium, including polarized ameloblasts (data not shown).

The transgenic mice showed a shaggy appearance (Fig. 2A and B). In two lines, whiskers (Fig. 2C and D), coat hairs (see Fig. 6A), and tail hairs (Fig. 2E and F) were curly. Macroscopically, the tails looked relatively normal (data not shown) and did not have kinks like the ectodysplasin null *Tabby* mice (Falconer, 1953). Scanning electron microscopy revealed that scale-like skin folds in the tail of transgenic mice were larger than in wild type (Fig. 2E and F). Nails of the adult animals were clearly longer than those of the control littermates, and the grooves of the toe skin, the plicae digitalis, were not as deep as in wild type (Fig. 2G). From 5 weeks onwards, the upper incisors started to overgrow due to excessive wearing of the lower incisors (Fig. 2H and I). When provided with soft diet, K14-*Eda-A1* transgenic founder animals or their F_1 progeny ($N = 10$) have lived for more than 1 year with no apparent skin tumors. Expression of ectodysplasin is regulated by canonical Wnt signaling mediated by Lef1 (Laurikkala et al., 2001, 2002). K14 promoter-driven expression of a stabi-
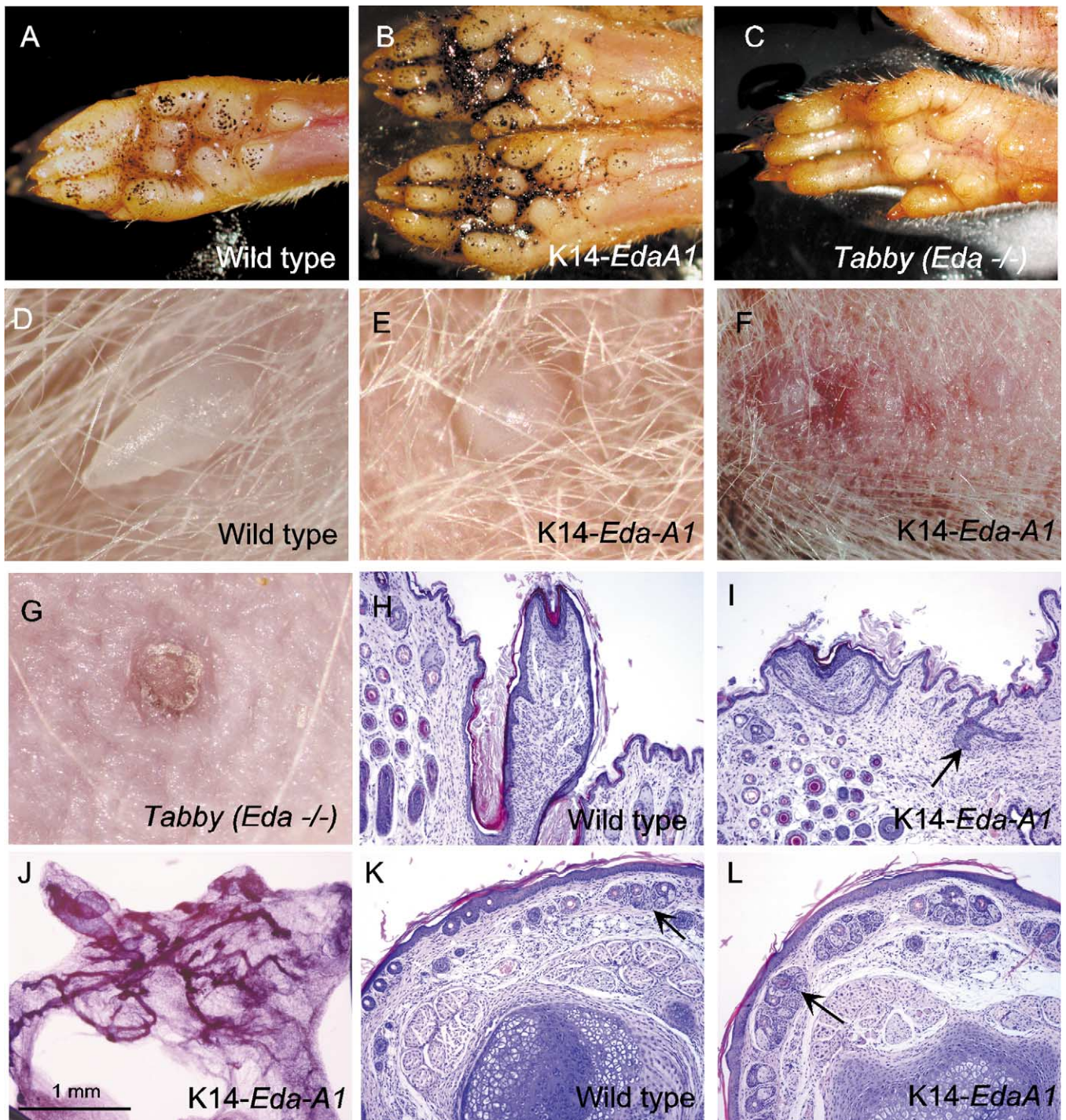
Fig. 3. Overexpression of *Eda-A1* stimulates the formation of eccrine glands. (A–C) Sweat glands were visualized by using iodine-starch staining. More staining, indicating increased sweat production, is apparent in the K14-*Eda-A1* foot pads (B) compared with wild type feet (A). *Tabby* mice have almost no staining (C). (D–J) The shape and number of the transgenic nipples are altered. Wild type nipples are high and tapering (D, H), whereas transgenic females have round nipples (E, F, I). Supernumerary nipples are seen along the former milk line in transgenic females. At the site of the third nipple, they are often abnormally close to each other (F). In tissue sections, rudimentary extra nipples are also observed (I, arrow). Some of the extra nipples of the transgenic animals are associated with a small mammary gland fat pad (J). *Tabby* females have abnormally flat and round nipples (G). (K, L) Sebaceous glands are larger in sections of the tail skin of K14-*Eda-A1* animals (arrows in K and L).

lized form of the Wnt mediator β-catenin, which mimics increased Wnt signaling, results in hair follicle tumors (Gat et al., 1998). The absence of tumors in K14-*Eda-A1* mice suggests that ectodysplasin signaling is not involved in the tumorigenic effects of continuously active β-catenin.
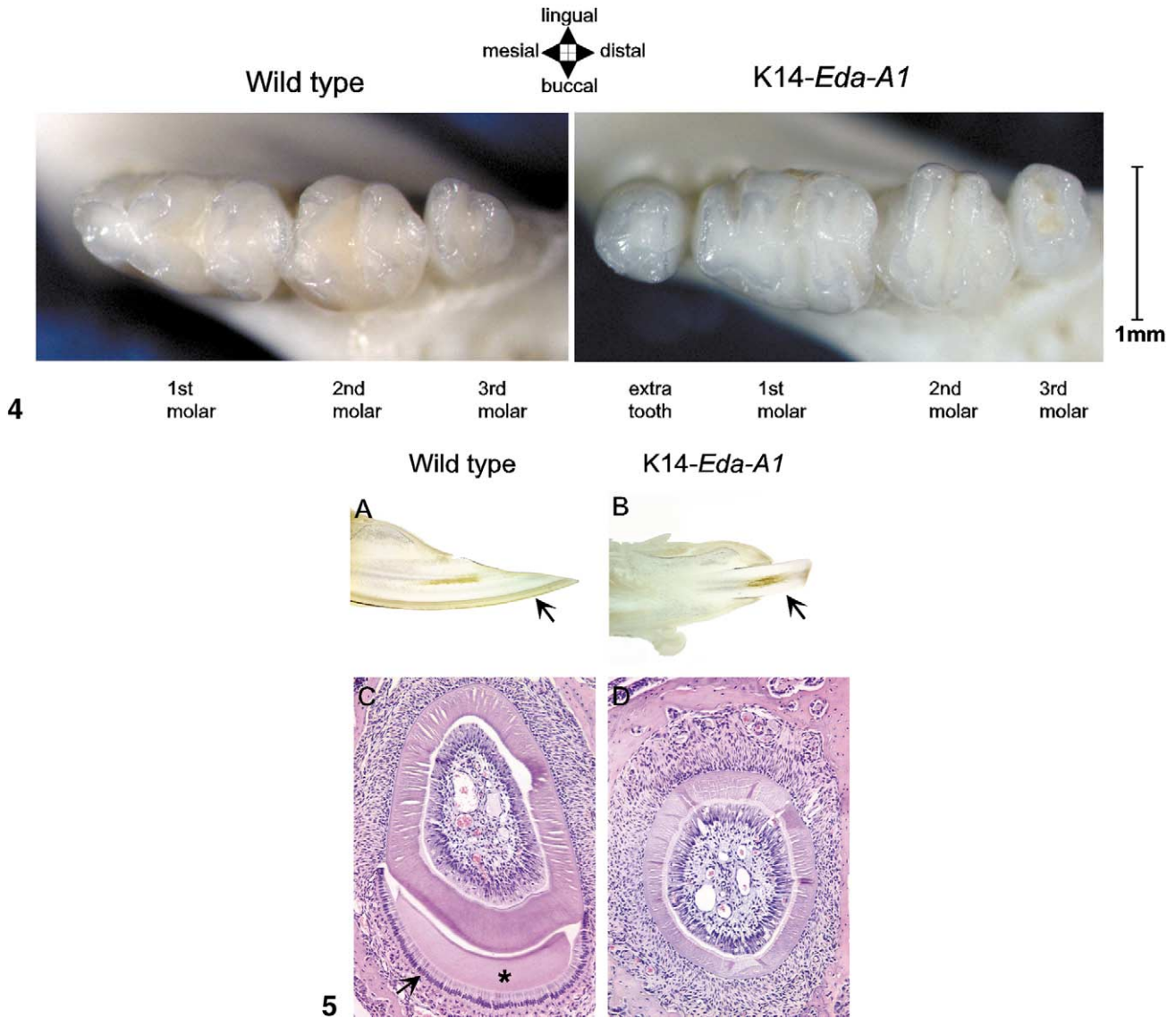
Several exocrine glands including sweat glands are hy-

Fig. 4. An extra tooth is induced and the shape of molars is changed in K14-*Eda-A1* jaws. Wild type mice have three molars at both sides of the lower jaw, but the K14-*Eda-A1* transgenic mice often have an extra tooth in front of the first molar as shown in the coronal view.

Fig. 5. K-14-*Eda-A1* incisors lack enamel. (A, B) Unstained plastic ground sections. (C, D) Hematoxylin–eosin-stained paraffin cross sections. Wild type mouse incisors have enamel (arrow in A) throughout the labial side, whereas the transgenic incisors are devoid of it (B). (C, D) Polarized ameloblasts (arrow) and enamel secreted by them (asterix) are visible in the wild type incisor (C) but are not detected in the K14-*Eda-A1* incisor (D).

poplastic or absent in the HED patients and *Tabby* mice. Mice have sweat glands only in their foot pads, where sweat formation can be visualized by applying iodine solution and starch–oil mixture (Wada and Takagaki, 1948). Sweat test on foot pads of transgenic, wild type, and *Tabby* mice revealed that transgenic mice had increased sweat production (Fig. 3B) as compared with wild type mice (Fig. 3A). The feet of *Tabby* mice lack sweat glands (Grüneberg, 1971), and as previously reported showed practically no staining (Fig. 3C; Blecher et al., 1990).

Normal mouse females have five pointed nipples, three thoracic and two inguinal, on each side of the abdomen (Hennighausen and Robinson, 2001; Fig. 3D and H). K14-*Eda-A1* transgenic females had supernumerary nipples along the pre-

sumptive milk line (Fig. 3F), and the shape of the nipples was abnormally round (Fig. 3E and I). Some but not all of the supernumerary nipples were associated with small fat pads (Fig. 3J). Nevertheless, these extra fat pads did contain epithelial branches and proliferated upon pregnancy (data not shown). The mammary glands of *Tabby* females have not been previously characterized, but human HED patients have been reported to have occasionally hypoplastic or absent nipples (Kere, 1998). Therefore, we analyzed the nipples of *Tabby* females and found them to be rudimentary and flat in appearance, albeit normal in number and location (Fig. 3G). Despite having abnormally formed nipples, *Tabby* females as well as most, although not all, *Eda-A1*-overexpressing females nursed their offspring normally.

Development of the sebaceous glands, exocrine glands associated with hair follicles, was also affected in K14-*Eda-A1* mice. In cross sections of the tail skin, the K14-*Eda-A1* transgenic tail hairs had bigger sebaceous glands than the wild type hairs (Fig. 3K and L). Also, the sebaceous glands of the back skin appeared larger than in wild type mice (see Fig. 7C and D).

K14-Eda-A1 mice had extra teeth

Mice normally have 1 incisor and 3 molars in each jaw quadrant. They thus lack cuspids and premolars and have a toothless diastema region between incisors and molars. K14-*Eda-A1* transgenic mice developed frequently an extra tooth in front of the first molar (Figs. 2I and 4). This tooth was smaller than the first and second molars and about the same size as the third molar. It had a round shape resembling premolars in other mammals, and the cusp pattern was not clear. Out of 15 adult individuals examined 10 had an extra tooth on at least 1 side of the mandible. Four of them had an extra tooth bilaterally, and 1 of these had a 4th tooth also in the upper jaw in front of the first molar. The molars of the transgenic mice were shorter and wider than in controls. The shortening was most pronounced in those rows that contained 4 teeth.

Ectodysplasin overexpression disturbed enamel formation

The overgrowth of the maxillary incisors was the first indication of enamel hypoplasia in K14-*Eda-A1* mice. The wearing of the incisors, which keeps the incisors sharp and is compensated by continuous growth of the teeth, was accelerated in the mandibular incisors so that they wore out during the first weeks of life. Macroscopic analysis indicated that the thickness of the incisors was reduced and that also molars were worn more rapidly. The histological analysis showed that adult transgenic incisors completely lacked the enamel, which is the hardest tissue of vertebrates. (Fig. 5). In plastic sagittal sections, the absence of enamel was evident along the whole length of the incisor (Fig. 5A and B). Demineralized paraffin sections indicated that well-organized polarized ameloblasts, normally secreting the enamel, were absent in transgenic incisors (Fig. 5C and D). There is some evidence that enamel is also affected in HED patients (Pirinen, 1998).

Hair types, length, and cycling are affected in the Eda-A1 overexpressing mice

Mice normally have four hair types in their pelage, which can be distinguished by their length and appearance (Fraser, 1951; Fig. 6A). Guard hairs (tylotrichs) are straight hairs of 9 mm or more in length that begin to develop at E14 (Mann, 1962). Awls are straight hairs shorter than 9 mm, and they develop as the second wave of hairs at E17. Auchenes are of similar length to awls but have one constriction appearing as a bend. Auchenes and zigzag hairs are last to develop, budding at the time of birth. Zigzag hairs have three to five bends and they form the soft undercoat of the animal.

The pelage hairs of all K14-*Eda-A1* transgenic mice were abnormal (Fig. 6A). Two of the lines had only curly hairs, whereas the other eight transgenic founder animals, and the three transgenic lines derived from them, had only straight hairs. Normally, the straight hairs, guard, and awl hairs, can be easily classified based on their length, as they form two distinct length groups which do not overlap (Fig. 6C). Transgenic mice with straight hairs were analyzed in detail. Hairs were found at all lengths from 5.5 to 13 mm, and no obvious length groups could be distinguished (Fig. 6C). If these hairs were classified according to the conventional criteria, so that hairs shorter than 9 mm were awls and hairs longer than 9 mm were guard hairs, the proportion of the guard hairs was dramatically increased in the transgenic pelage (Fig. 6C). Hair types can also be distinguished based on the internal structure of their medulla, which differs in the number of longitudinal rows of air-cells seen under light microscope (Falconer et al., 1951; Sundberg, 1994). Guard hairs have two rows of air cells, whereas awls have two, three, or even four of them. All transgenic straight hairs looked relatively similar regardless of their length but differed from normal guard and awl hairs (Fig. 6B), thus leaving their identity unclear. No auchenes or zigzags were found in transgenic animals (Fig. 6D), so they lacked the undercoat, which may have caused the shaggy appearance of transgenic mice.

Hair growth involves the unique phenomenon of cycling. The active growth phase (anagen) is followed by rapid apoptosis-driven regression phase (catagen) before the follicles enter the resting phase (telogen) (Muller-Rover et al., 2001). The fact that *Eda-A1* transgenic mice had abnor-

Fig. 6. Overexpression of *Eda-A1* disrupts hair type composition. (A) Wild type mouse pelage consists of four types of hairs: guard hairs (g), awls (awl), zigzags (zz), and auchenes (au). K14-*Eda-A1* hair composition is abnormal; in most transgenic lines, all hairs are straight, and in two lines, they are all curly. (B) Internal structure of wild type (wt) guard (gh) and awl hair types and a transgenic hair (K14-*Eda-A1*) shown by light microscopy. The air cell organization of the transgenic hair is different from both wild type hairs. A total of 38 transgenic hairs were analyzed. (C) Comparison of the lengths of wild type straight hairs ($n = 235$), awls and guard hairs, and K14-*Eda-A1* straight hairs ($n = 399$). Wild type awl hairs are between 5 and 8 mm long with the majority of the hairs being 6.5–7.5 mm long. The guard hairs counted are all 11.5 mm. On the contrary, all hair lengths between 5.5 and 13 mm are found in the transgenic straight hairs. (D) The proportions of different hair types are affected in the transgenic pelage. A total of 54.2% of wild type hairs are zigzags, 40.2% awls, 3.3% guard hairs, and 2.4% auchenes. The straight-haired K14-*Eda-A1* mice have only hairs of awl type, 33.2%, or guard hair type, 66.8%. Wild type, $n = 445$; transgenic, $n = 399$. Classification of transgenic straight hairs was based on the length of hairs: hairs shorter than 9 mm were classified as awls, and hairs 9 mm or longer as guard hairs.
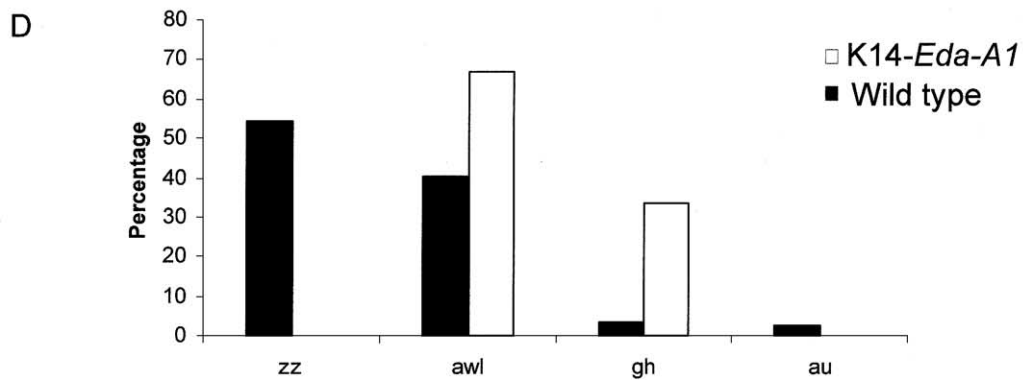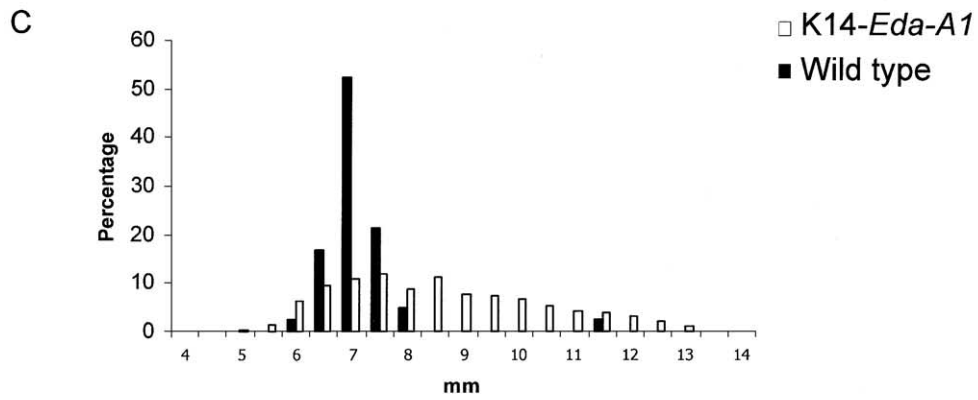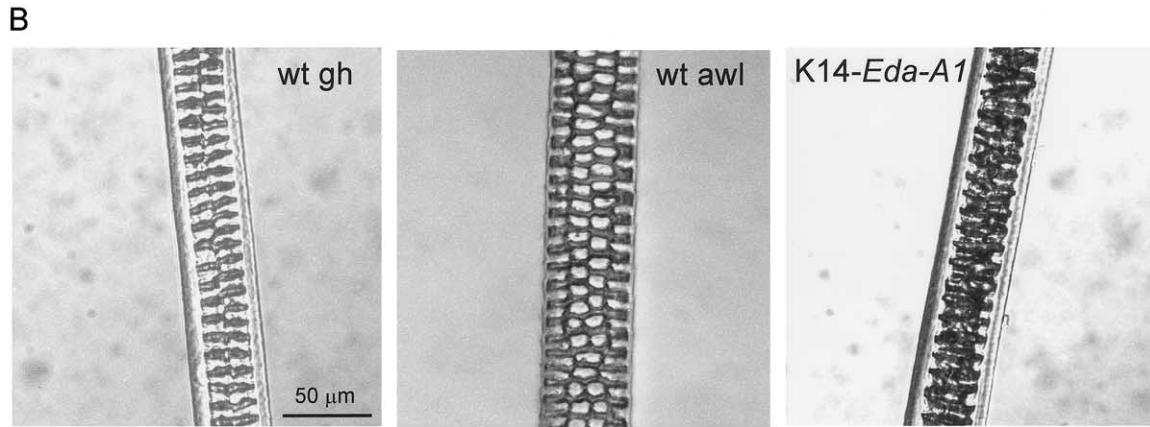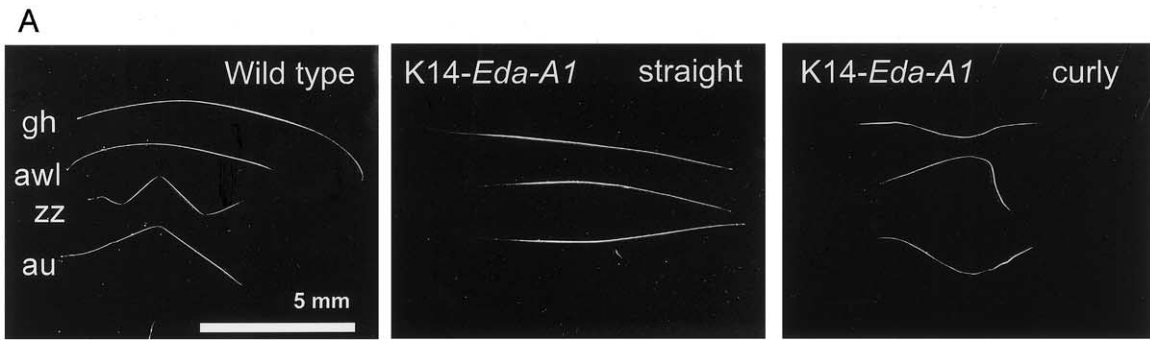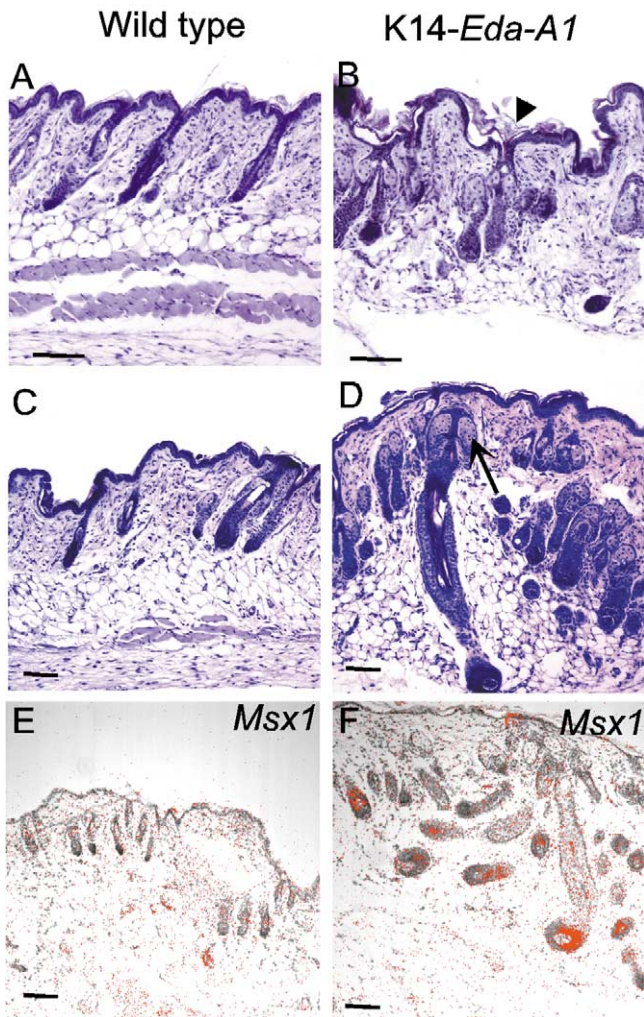
Fig. 7. The first anagen phase of K14-*Eda-A1* mice hair cycle is prolonged. (A, C) At P21, hair follicles in wild type dorsal skin are at telogen, as visualized by their retraction into the dermis and the degeneration of the hair bulb. (B, D) In contrast, the transgenic hair follicles still have well formed bulbs, indicating that they are at anagen or early catagen stage. Also, the sebaceous glands are bigger (arrow in D), and some of the follicles are abnormally close to each other, even sharing the upper part of the hair canal (arrowhead in B). (E, F) *Msx1* is expressed in the transgenic hair bulbs, whereas it is downregulated in the degenerate wild type hair bulbs. Transgenic skin also contained more big guard hair type of follicles and the follicles were disoriented (D, F). Scale bar, 100 μm.

mally long hairs suggested that the cycling of the follicles might be affected. FGF5-deficient *angora* mice have unusually long hairs due to a prolongation of anagen (Hebert et al., 1994). We compared hair follicles of transgenic and wild type littermates during the highly synchronized first hair cycle. At P21, hair follicles of wild type mice had retracted and the entire hair follicle resided in the dermis (Fig. 7A, C, and E), indicating that the follicles had entered telogen (Muller-Rover et al., 2001), whereas the transgenic follicles still had well-formed bulbs characteristic of anagen/early catagen (Fig. 7B, D, and F). Transgenic hair follicles, which were localized close to each other, or had been fused, also had abnormal orientation (Fig. 7B and D).

Transcription factor *Msx1* is expressed in the germinative matrix of the anagen follicles, but its expression is down-regulated upon catagen onset (Satokata et al., 2000). Accordingly, no *Msx1* expression was detected in wild type hair bulbs at P21, whereas a prominent expression was observed in the bulbs of the transgenic hair follicles (Fig. 7E and F). Thus, the wild type and *Eda-A1* overexpressing hair follicles were at different stages of hair cycle at P21, the first anagen being longer in the transgenic skin.

Hair placodes were produced continuously in the Eda-A1 overexpressing embryos

Next, we examined the development of hair follicles in hematoxylin–eosin-stained paraffin sections from E14 until birth (Fig. 8, and data not shown). Because the time of formation of each hair follicle depends on the body location (Mann, 1962), we took care in choosing sections from corresponding locations for a comparative analysis. Guard hair follicles were evident both in wild type and Eda-A1 transgenic mice at E14 (data not shown). Already at E15, new hair follicles were emerging in transgenic skin (Fig. 8B), a phenomenon not observed in wild type skin (Fig. 8A) where the development of the next follicles (producing awls) starts at E17. The newly formed follicles of *Eda-A1*-expressing mice were abnormally close to the previously formed ones (Fig. 8A–D), and sometimes the follicles even fused (Fig. 8F), and later they shared the upper portion of the hair canal (Fig. 7B). Fused follicles could be seen quite regularly in transgenic mouse skin, especially in the curly-haired lines, but not in the wild type skin (Figs. 7A and 8E). Thus, it appeared that in transgenic mice the hair follicles were not produced in separate first (guard) and second (awl) waves as in the wild type mice; instead, new placodes were observed continuously between E14 and birth. In situ hybridization of P2 transgenic skin samples with a probe specific for the Eda-A1 receptor *Edar*, which is expressed in the forming placodes and later in the bulbs of the follicles, revealed that there were follicles of all developmental stages and they were located close to each other (Fig. 8G and H). During early development (E11–E13), *Edar* is expressed throughout the epithelium and is among the first genes to be upregulated in the developing placodes at E14, while in interfollicular epithelium, its expression is downregulated (Huelsken et al., 2001; Laurikkala et al., 2002). Overexpression of *Eda-A1* in the entire epithelium did not cause an upregulation of the interplacodal expression of *Edar* (Fig. 8G–J). Contrary to the defective development of skin appendages, the epidermis itself had a normal histological appearance in all stages analysed (Figs. 7 and 8).

Discussion

We have shown that the overexpression of ectodysplasin-A1 affects multiple stages in the formation of several
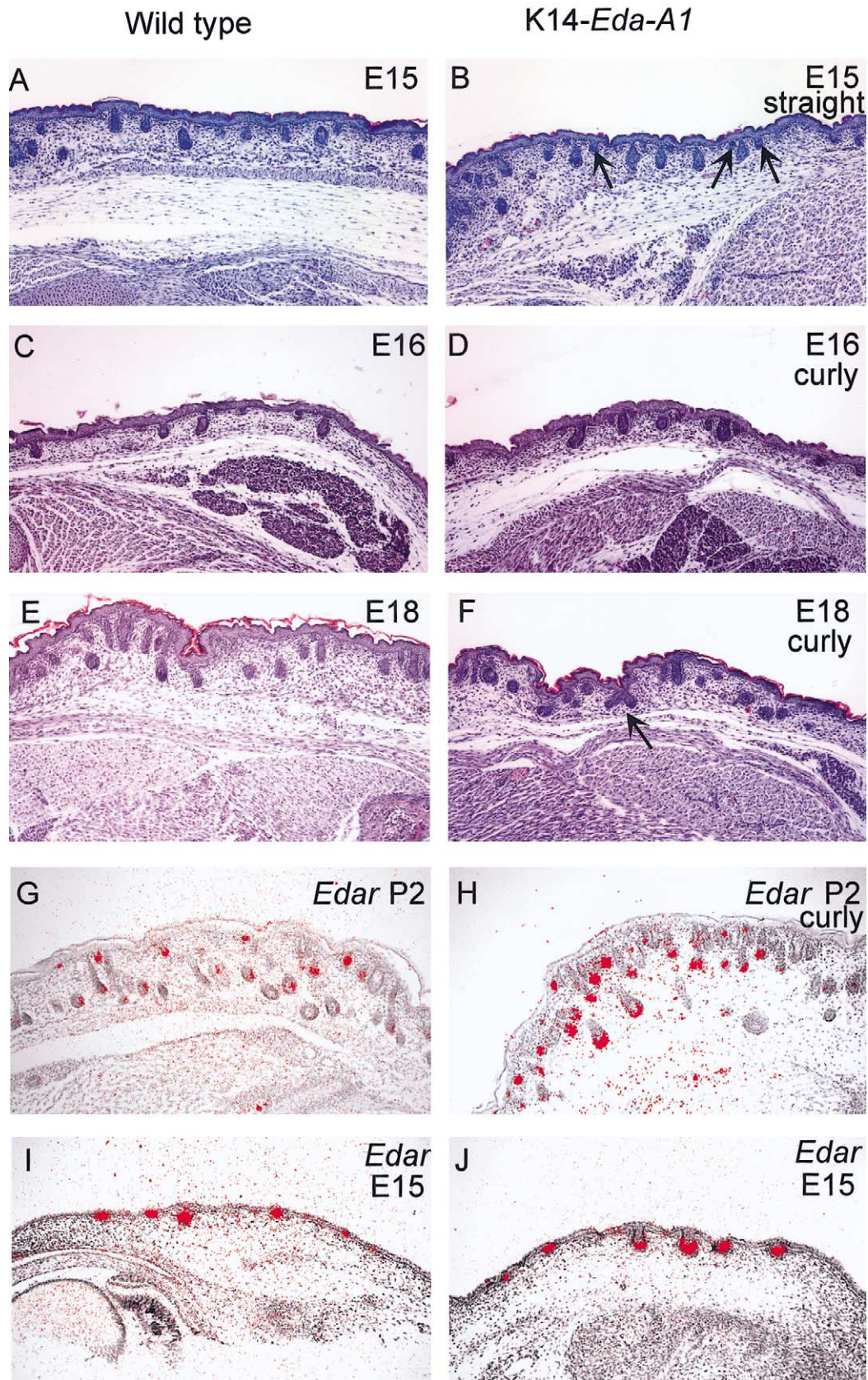
Fig. 8. Continuous formation of hair follicles in K14-*Eda-A1* developing skin. (A–F) Hematoxylin–eosin-stained paraffin sections of dorsal skin. The first wave of follicle development (guard hairs) is visible in both wild type and transgenic skin between E15 and E18 (A–F), but in the transgenic skin, new hair buds continue to emerge (arrows in B and D). Occasionally, fusion of hair follicles is seen (arrow in F). (G, H) At P2 in wild type head skin, *Edar* expression is seen both at the developmentally more advanced follicles (guard hairs and awls) and at the forming placodes (the latest forming zigzags and auchenes). In the transgenic skin, *Edar*-expressing follicles are found at a wider range of developmental stages. (G, H) At E15, *Edar* is expressed in the hair placode but downregulated in the interfollicular region of both wild type (I) and transgenic (J) head skin.

ectodermal organs, such as hairs, teeth, nails, and various glands, including mammary, sebaceous, and sweat glands. In general, organogenesis was stimulated in the K14-*Eda-A1* transgenic mice. They had extra teeth and mammary glands, their hairs and nails grew longer than normal, sweat gland function was stimulated, and sebaceous glands were large. An unexpected finding was the inhibition of enamel formation in incisor teeth, which resulted in their rapid wearing. Our results suggest that ectodysplasin–Edar signaling has a central function in the formation of most, or perhaps all organs, which form as ectodermal appendages. This function appears to have been conserved in evolution since *Edar* mutations cause a scaleless phenotype in Medaka fish (Kondo et al., 2001). The results of the present study also indicate that the level of the Edar receptor is not a limiting factor in ectodysplasin signaling as was suggested recently based on the analysis of transgenic mice overexpressing *Eda-A1* under the CMV promoter (Srivastava et al., 2001). This transgene rescued partly the *Tabby* phenotype, but no alterations were found when it was expressed in wild type mice. The different findings are probably explained by differences in the amount of transgene expression in the two studies.

Overexpression of the *Eda-A2* splice form under the K14 promoter did not cause a detectable phenotype. This is conceivably due to the difference in receptor binding between the two Eda splice forms. Only Eda-A1 binds to Edar, whereas Eda-A2 uses another TNF receptor, Xedar (Yan et al., 2000). The function of Xedar is at present unknown. Its expression in the epidermis and hair follicles begins later than the expression of *Edar* (E16), and therefore it was rather unexpected that Eda-A2 increased the number of hair follicles in cultured skin explants (dissected at E13.5–E14.5) in vitro (Yan et al., 2000), an effect not found in our transgenic mice. The expression patterns of the Eda-A1 and Eda-A2 splice forms are not known except during hair development, where Eda-A2 is detected starting at E17 in the bulb region of the already formed follicles (Yan et al., 2000), suggesting that it does not regulate placode formation in vivo.

Ectodysplasin-A1 regulates the length, structure, and cycling of hairs

The hairs in K14-*Eda-A1* mice were abnormal in all regions analyzed, including the pelage, tail hairs, and vibrissae. Both the structure and shape of hairs were affected. All hairs were either curly or straight, and the pelage hair types with bends, the auchenes and zigzags, were missing. We attempted to define the identity of the transgenic hairs by microscopical analysis, which reveals the fine structure of the hair shaft. However, they did not display the characteristic features of any of the four mouse hair types, which normally develop in three separate waves, although they showed most resemblance to guard hairs. Also, in *Tabby/downless/crinkled* mice, the fine structure of the awls that

develop is disturbed (Falconer et al., 1951; Pispa et al., unpublished results). It is not known how the production of the hair shaft differs in the separate waves of hair follicles in wild type mouse embryos, but our results suggest that the levels of activities of various signaling molecules including ectodysplasin may regulate this process.

The hairs of the K14-*Eda-A1* mice were significantly longer than wild type hairs. We showed that the growth phase (anagen) of the first hair cycle was prolonged in the transgenic mice. As the length of hairs is usually proportional to the duration of the anagen phase (Muller-Rover et al., 2001), we suggest that the longer hairs in the transgenic mice resulted from a defect in hair cycling. The expression of many molecules oscillate during different phases of the hair cycle (Muller-Rover et al., 2001); however, only a few genes, such as *Fgf5* and *Bcl-x$_L$*, have been shown to directly influence hair cycling (Hebert et al., 1994; Pena et al., 1999). It is possible that hair formation was also stimulated by alterations in the proliferation or differentiation of hair matrix cells as shown in Wnt3 overexpressing mice (Millar et al., 1999) since both the Edar receptor and the transgene were expressed in the hair bulb.

Ectodysplasin-A1 can initiate ectodermal organ development

Transgenic mice overexpressing Eda-A1 had supernumerary teeth and mammary glands. To our knowledge, this is the first example of an experimental mouse model with such a phenotype. As *Edar* is expressed in the dental placodes, we have suggested earlier that ectodysplasin–Edar signaling regulates their formation and/or function (Laurikkala et al., 2001). Our present data suggest that increased Edar signaling within the ectoderm is sufficient for the initiation of not only teeth but also other ectodermal organs. However, it appears that Edar signaling is redundant with some other TNF pathway or different molecular mechanisms may compensate for its function because ectodermal organ development is inhibited only partially when the pathway is totally blocked in human HED patients and the corresponding mouse mutants *Tabby, downless,* and *crinkled.*

Extra teeth formed frequently in front of the first molars in the K14-*Eda* mice. In *Tabby* mice (*Eda* loss-of-function mutants), tooth number is reduced as the third molars or incisors are commonly lacking (Grüneberg, 1965). It is puzzling that *Tabby* heterozygotes occasionally have extra teeth in the same location as the ectodysplasin overexpressing mice (Grüneberg, 1966). Teeth develop from the dental laminae, which are thickenings of the ectoderm lining embryonic facial prominences and mark the locations of future dental arches. The placodes of the incisor and molar teeth form from the anterior and posterior parts of the dental lamina, respectively, and the buds of the first, second, and third molars develop sequentially in the molar field. Two models may explain the effects of increased Edar signaling.

In the first model, formation of an extra tooth is due to an early function of Edar (high transgene expression was detected as early as at E10), resulting in increased size or signaling activity of the molar field. In the second model, increased Edar signaling from the first molar placode induces the formation of an ectopic tooth next to it.

Because of similarities between early mammary gland and tooth development, the supernumerary mammary glands observed in the K14-*Eda* mutants conceivably have a similar pathogenetic mechanism as the extra teeth. Mammary glands form from placodes in the milk line which resembles morphologically and functionally the dental lamina (Propper, 1978). Increased signaling within the mammary line could lead to the formation of several buds as suggested above for the teeth. It is notable that these extra organs did not develop outside the dental lamina or mammary line, indicating that Edar signaling does not affect the regional specification of the ectoderm. Instead, it seems to activate a preexisting potential that resides in these specified areas which is not used during normal mouse development. We are presently analyzing the dynamics of the formation of tooth and mammary gland placodes and their budding to resolve the pathogenesis of the observed phenotypes.

Edar is coexpressed with ectodysplasin in the simple ectoderm prior to hair placode formation, and as placodes are initiated, it is downregulated in the interfollicular ectoderm and upregulated in the placodes. Ectodysplasin is required for this patterning of *Edar* expression to the placodes and this likely involves reciprocal epithelial–mesenchymal interactions (Laurikkala et al., 2002). In K14-*Eda-A1* embryos, *Edar* expression was patterned to the primary hair placodes and downregulated in the interfollicular epidermis (Fig. 8I and J) like in wild type mice (Laurikkala et al., 2002), and the initiation of the first wave of hair follicles was apparently normal, since no alterations were observed in their timing. However, thereafter, new follicles developed continuously abnormally close to preexisting ones, suggesting that Edar signaling negatively regulates lateral inhibition of placode fate in surrounding cells. Thus, most likely, the effects of ectopic Eda expression in K14-*Eda-A1* mice on the initiation of placodes were indirect.

The patterning and growth of hair and feather placodes is regulated by an interplay of activators, such as FGFs and inhibitors such as BMPs (Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999; Jiang et al., 1999). The role of BMP2 and BMP4 in mediating lateral inhibition during feather tract development is well established (Jung et al., 1998; Noramly and Morgan, 1998). Accordingly, application of noggin, a potent inhibitor of BMP2/4, on cultured murine embryonic skin resulted in a marked increase in the number of induced hair follicles (Botchkarev et al., 1999). We do not know at present how increased Edar signaling in the placodes results in precocious initiation of new placodes. A similar phenotype has been observed in chicken by forced activation of the β-catenin signaling pathway, which resulted in ectopic feather buds in interfollicular regions (Noramly et al., 1999). Also, expression of a stabilized form of β-catenin using K14 promoter induced ectopic hair follicles but only during postnatal hair cycling (Gat et al., 1998). In accordance with these results, no hair placodes are formed in mice overexpressing Dickkopf 1, an inhibitor of the canonical Wnt pathway (Andl et al., 2002). It seems that both Edar and Wnt pathway act very early in follicular morphogenesis and can both promote placode formation. We suggest that Edar signaling regulates the delicate balance between activators and inhibitors of placode formation and overstimulation of placodal Edar changes this balance so that more activator(s) is made.

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