Ectodysplasin-A1 Promotes Epithelial Branching and Duct Formation in Developing Submandibular Glands

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Purpose: The genes encoding ectodysplasin-A (*ED1/Eda*) are involved in development of ectodermal organs and their mutations cause human and murine X-linked ectodermal dysplasia, XLHED and *Tabby*, respectively. In the present study, the effects of absent Eda signalling on human salivary gland function, as well as absent and ectopic signalling of the splice form Eda-A1 on mouse submandibular gland (SMG) morphogenesis and transcription of selected signalling molecules were examined.

Material and methods: Ten male patients diagnosed with XLHED as well as ten age-matched male control persons were included in the study. These persons were extracted from a larger group of persons diagnosed with different forms of ectodermal dysplasias. All participants were examined with regard to unstimulated and chewing stimulated whole salivary flow and citric acid stimulated parotid and submandibular/sublingual flow.

Results: The study demonstrated that salivary secretion is reduced in persons diagnosed with XLHED. *Tabby* SMGs were found to have few and small ductal structures, whereas SMGs in K14-*Eda-A1* transgenic mice overexpressing the *Eda* splice form *Eda-A1* were dysplastic with large ductal structures. Early SMGs of K14-*Eda-A1* transgenic mice had more branches than wild type control explants, and this was still the case after 44 h in culture. *Eda-A1* expression was observed in wild type SMG mesenchyme and in both mesenchyme and epithelium in K14-*Eda-A1* transgenic SMGs. *Eda-A1* clearly promoted the formation of the luminal epithelium expressing *Tsc-22*.

Conclusions: These findings indicate that Eda-A1 has important functions during both human and murine salivary gland development.

Key words: Ectodysplasin-A1, X-linked hypohidrotic ectodermal dysplasia, Tabby, submandibular gland, Edar, TSC-22

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INTRODUCTION

Development of several ectodermal organs in vertebrates depends on signalling by ectodysplasin (Eda), its receptor Edar and the cytoplasmic Edar binding death domain adapter protein, Edaradd (Mikkola and Thesleff, 2003). These signalling molecules have been discovered through the cloning of genes mutated in human and murine hypohidrotic ectodermal dysplasias (HED). These conditions are characterised by abnormal development of hair, teeth and exocrine glands, in particular sweat glands (OMIM). There is growing evidence that also salivary glands are affected by hypohidrotic ectodermal dysplasias. A decreased salivary secretion in persons diagnosed with the condition has been mentioned in the literature (Soderholm and Kaitila, 1985; Clarke et al, 1987), but we are not aware of any publications regarding salivary gland function in HED *per se*.

However, some studies regarding salivary gland morphology and development in *Tabby* mice have been published (Blecher et al, 1983; Jaskoll et al, 2003).

The human X-linked hypohidrotic ectodermal dysplasia (XLHED) is caused by mutations in the gene encoding Eda (*ED1*). Also the *Tabby* phenotype results from a mutation in the corresponding gene, *Eda*, located on the X-chromosome. The Eda protein is the only member of the TNF superfamily that contains a collagen-like region immediately preceding the most C-terminal TNF domain. The TNF domain, the collagen-like domains, and a furin consensus cleavage site required to produce a soluble signalling molecule are all important for the biological function of Eda (Thesleff and Mikkola, 2002a; Mikkola and Thesleff, 2003). Two functional isoforms of Eda, Eda-A1 and Eda-A2 have been described (Srivastava et al, 1997; Bayes et al, 1998; Mikkola et al, 1999). Eda-A2 differs from Eda-A1 by the absence of two amino acids in the TNF domain and the ligands have distinct receptors. Eda-A1 binds to Edar, whereas Eda-A2 binds to another TNF receptor called Xedar (Yan et al, 2000). It has been shown that transgenic expression of Eda-A1 in *Tabby* mice can rescue the hair and sweat gland phenotype (Srivastava et al, 2001). Furthermore, a recent study demonstrated a reversion of the *Tabby* phenotype after treatment with recombinant Eda-A1 in pregnancy (Gaide and Schneider, 2003). Overexpressing Eda-A1 in developing mouse ectoderm resulted in alterations in a variety of ectodermal organs, whereas Eda-A2 overexpression did not cause a detectable phenotype (Mustonen et al, 2003). These findings indicate that the Eda-A2/Xedar pathway is less important in the development of ectodermal organs. Eda-A1/Edar signalling activates NF-κB (Yan et al, 2000; Koppinen et al, 2001; Kumar et al, 2001), but little is known of downstream effector molecules. Interactions between epithelium and mesenchyme are instrumental in the regulation of ectodermal organ morphogenesis. The initial organ-buds express a variety of signalling molecules in a strictly regulated cascade that coordinate and regulate the inter-tissue cross-talk necessary for organ development. Many of the molecules involved are shared between different organs (Millar, 2002; Thesleff and Mikkola, 2002b; Pispa and Thesleff, 2003) and may potentially be regulated directly or indirectly by events originating in the Eda/Edar pathway. These molecules include Eda as well as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), sonic hedgehog (Shh), and Wnts.

In mouse, submandibular gland (SMG) development is initiated at embryonic day 11. After initial budding, development proceeds by branching morphogenesis, involving repeated interactions between the ectodermal epithelium and the adjacent mesenchyme (Bernfield et al, 1984; Denny et al, 1997), causing division and branching of the bud into a compound branched structure (Jaskoll and Melnick, 1999). Adult *Tabby* mice have been reported to have smaller SMGs than wild type controls, with delayed granular convoluted tubule development and reduced granular convoluted tubule proportion of the gland (Blecher et al, 1983). The role of Eda/Edar signalling in mouse SMG development has been studied in more detail recently. *Edar* mRNA expression was seen already at E12-E14 in the budding and branching SMG epithelium (Pispa et al, 2003). At later developmental stages both proteins were immunolocalized to apical surfaces of lumen bounding epithelium (Jaskoll et al, 2003). Postnatal *Tabby* SMGs were found to be hypoplastic, whereas *downless* (*dl*, loss of function of Edar) SMGs were severely dysplastic. Both postnatal *Tabby* and *dl* SMGs produced less total protein and mucin protein than controls, indicating that the Eda/Edar pathway also are important for histodifferentiation (Jaskoll, 2003). *In vitro* studies revealed that Eda-A1 supplementation to E14 SMG cultures caused an increase in branch number whereas inhibition of Eda/Edar signalling resulted in the opposite (Jaskoll et al, 2003).

The aims of the present study were to examine the effect of absent Eda-A1 signalling on salivary gland function and development as well as the effect of its ectopic mRNA expression on murine SMG development. Specific aims were: 1) To examine salivary gland secretory rates in persons with XLHED to see if the human condition affects salivary gland function; 2) To examine the embryonic and adult SMG morphology in *Tabby* mice, transgenic mice with ectopically expressing *Eda-A1* mRNA under the K14 promoter in the developing SMGs (K14-*Eda-A1* mice), and in wild type controls; 3) To analyse the branching morphogenesis of K14-*Eda-A1* SMGs *in vitro*; and 4) To examine the expression of selected signalling molecules in embryonic SMGs of wild type, *Tabby*, and K14-*Eda-A1* mice.

MATERIAL AND METHODS

Salivary Secretory Rates in Human XLHED

Ten male patients diagnosed with XLHED as well as ten age-matched male control persons were included in the study. These persons were extracted from a larger group of persons diagnosed with different forms of ectodermal dysplasias (Nordgarden et al, 2001a). All par-

ticipants were examined with regard to unstimulated and chewing stimulated whole salivary flow and citric acid stimulated parotid and submandibular/sublingual flow. The study protocol was evaluated and recommended by the Regional committee for medical research ethics in Norway (region II) and informed consent was obtained from all participants. If they were under 16 years of age, informed consent was obtained from both the child and his parents.

Morphological Evaluation of Embryonic and Adult SMGs

A *Tabby* mouse strain ($Eda^{Ta\text{-}6J}$) was obtained from Jackson Laboratories (Bar Harbor, ME, USA). The generation of K14-*Eda-A1* mice has been described elsewhere (Mustonen et al, 2003). All mice were maintained in accordance with the animal Welfare Act of 20 December 1974. Pregnant *Tabby* females were anaesthetized on day 15 (E15, n=3) and on day 18 (E18, n=3) of gestation with CO_2 and euthanized by cervical dislocation. The embryos were immediately collected and SMGs from 33 E15 and 26 E18 embryos were dissected out in phosphate buffered saline (PBS) under a stereomicroscope. The remains of the embryos were kept frozen for genotyping (see below). The embryological SMGs were fixed in 4% carnoy fixative overnight, dehydrated in alcohol, embedded in paraffin, and serially sectioned. The sections were stained with hematoxylin-eosin using standard procedures and examined in a light microscope.

E15 embryos (n=10) were collected from one and E18 embryos (n=31) from three female K14-*Eda-A1* transgenic mice and their SMGs dissected as described for *Tabby* mice. The embryonic SMGs were fixed in 4% paraformaldehyde overnight and processed as described above. K14-*Eda-A1* transgenic embryos were identified by the overtranscription of *Eda* mRNA in skin samples as demonstrated by *in situ* hybridisation (Mustonen et al, 2003). Adult SMGs from the female mice (n=3) demonstrating a typical K14-*Eda-A1* phenotype were collected at the same time the embryos were collected. Also adult SMGs from female wild type FVB/N mice (n=3) were collected and used as controls. The SMGs were collected in PBS, fixed in 4% paraformaldehyde overnight and processed as described above.

Genotyping of Tabby Embryos

In order to genotype the *Tabby* embryos, DNA was isolated from all of them using the QIAamp DNA Mini Kit as described by the manufacturers (QIAGEN AS, Norway). The Amplitaq Gold PCR Master Mix (Applied Biosystems, Norway) was used for PCR amplification of the Eda gene. PCR amplifications were carried out in 50 μl reaction volumes containing 1 μl DNA template, 50 nmol each primer and 25 μl PCR Master Mix. Reactions were heated to 95°C for 5 min, followed by 35 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 60 s and a final hold at 72 °C for 7 min. Forward and reverse primers used for the amplification of *Ta* exon 1 were 5′-CAG ATA GTG GTT GTC TCT GGA G-3′ and 5′-AAC AAC CTG ACC TGG ACA ACC TCT-3′ respectively (Srivastava et al, 1997). Mutations in the *Tabby* gene were determined by direct sequencing using the reverse primer (Eurogentec, Belgium). After purification, PCR products were submitted to sequencing using the Dye Terminator Cycle-Sequence ready Reaction DNA Sequencing Kit (Perking Elmer, Belgium). The sequence analysis was performed in an automated 377 DNA Sequencer (Perking Elmer, Belgium). Nucleic acid sequence similarity searches were performed manually.

Tissue Culture

Eleven E13.5 embryos from one mouse demonstrating the K14-*Eda-A1* transgenic phenotype were collected and their SMGs dissected in PBS under a stereomicroscope. The 22 dissected SMGs were then cultured in a Trowell-type of tissue culture as described earlier for tooth explants (Aberg et al, 1997). Cultures were photographed after 6 h, 20 h, 30 h and 44 h. At each time the branches were counted and the branch ratio (terminal bud number after 44 hours/initial bud number) was determined for each explant. K14-*Eda-A1* positive tissue samples were detected by whole mount *in situ* hybridisation using a riboprobe for Eda (Laurikkala et al, 2001).

In Situ Hybridisation

Paraffin sections obtained as described above were used for the K14-*Eda-A1* SMGs. In addition five E15 *Tabby* embryos were dissected from one mouse. The SMGs from the adult *Tabby* female were dissected out at the same time. These glands were processed as described for embryonic and adult K14-*Eda-A1* SMGs. Radioactive *in situ* hybridisation with 35S (Amersham Biosciences, England) labelled riboprobes on the paraffin sections was performed as described in Wilkinson and Green (1990). The following probes were used: Murine Eda and Edar (Laurikkala et al, 2001), murine Bmp4 (Vainio et al, 1993), murine p21 (Jernvall et al, 1998), murine Ptc (Kim et al, 1998), murine Fgf10 (Bellusci et al, 1997), and murine Tsc-22 (Dohrmann et al, 1999).

Table 1 Mean salivary secretory rates (ml/min±std) in persons with XLHED and healthy age-matched control persons

UWS	P	SWS	P	SS	P	PS	P	
XLHED (n=10)	0.14±0.1	< 0.001	0.61±0.51	0.001	0,37±0.29	0.02	0.56±0.33	0.15
Controls (n=10)	0.5±0.16		1.74±0.67		0.83±0.47		0.83±0.14	

UWS = Unstimulated salivary flow rate; SWS = chewing stimulated salivary flow rate; SS = submandibular salivary flow rate; PS = parotid salivary flow rate.

Statistical Analysis

The numerical data are presented as means±std, and the student t-test were used to examine differences between groups. A significance level of 5% was used.

RESULTS

Salivary Secretory Rates in Human XLHED

Unstimulated and chewing stimulated whole salivary as well as submandibular salivary flow was significantly reduced in the persons with XLHED as compared to controls. The parotid flow rate was also lower in the XLHED persons compared to the control persons. However, this last finding was not statistically significant (Table 1).

SMGs in Tabby Mice have fewer Epithelial Branches than Wild Type SMGs

Mice demonstrating the $Eda^{Ta\text{-}6J}$ phenotype have a single base deletion at position+550 of the coding region in exon 1 of the *Eda* gene. This results in a frame shift that produces a truncated, inactive protein product (Srivastava et al, 1997). Five E15 and three E18 embryos, had the single base deletion responsible for the *Tabby* phenotype in this strain as demonstrated by sequence analysis. The sequence analysis was inconclusive in four E15 and eight E18 embryos, and these mice were not included in the morphological evaluation. Littermates with wild type *Eda* sequences were used as controls.

Morphological evaluation of the *Tabby* E15 and E18 SMGs revealed that they generally had fewer epithelial branches separated by more mesenchymal cells than wild type SMGs (Figs. 1A, B, C, D). At the time points studied *Tabby* and wild type SMGs had reached the same level of development, namely the canalicular stage in E15 glands and the end bud stage in E18 glands (Jaskoll and Melnick, 1999).

Tabby adult mice demonstrated the typical phenotype; yellow coat, absence of hair on ears and tail, a bald patch behind the ears and lack of guard hairs. Fewer and smaller ducts were observed in adult *Tabby* glands than in wild type (Figs. 1G, H).

SMGs of K14-Eda-A1 Mice are Dysplastic

Tissue sections of dissected SMGs at E15 and E18 showed that both the wild type and the K14-*Eda-A1* SMGs had reached the same levels of development at these stages; the canalicular stage at E15 and the end bud stage at E18. However, the forming ducts in the K14-*Eda-A1* transgenic SMGs were larger than in wild type at both E15 and E18 (Figs. 1B, C, E, F).

Adult K14-*Eda-A1* mice have a distinct phenotype, including abnormal hair resulting in a "shaggy" appearance, long nails, supernumerary nipples and teeth, and disturbed enamel formation (Mustonen et al, 2003). Here, SMGs from adult K14-*Eda-A1* mice were dissected and found to be severely dysplastic demonstrating abnormally large ductal structures and large blood vessels (Fig. 1I).

Developing SMGs of K14-Eda-A1 Embryos are more Branched than Wild Type SMGs

Since the adult K14-*Eda-A1* SMGs had enlarged ducts and since *in vitro* studies have shown that Eda-A1 supplementation increased branching in developing SMGs (Jaskoll et al, 2003), the early appearance of K14-*Eda-A1* SMGs cultured *in vitro* was examined here. Of the 11 foetuses collected, 6 (12 glands) were positive for the transgene as demonstrated by whole mount *in situ* hybridisation. The remaining five (10 glands) littermates were used as controls. The numbers of developing branches were counted on the photographs taken after 6 h, 20 h, 30 h and 44 h. Statistical analysis revealed that SMGs from K14-*Eda-A1* transgenic mice had more branches at all time points studied, although this finding was only borderline significant at E13+6 h. Also the development of new branches (branch ratio) proceeded faster in the K14-*Eda-A1* transgenic glands as compared to controls, although this finding was not significant at the chosen level (Table 2, Fig. 2).

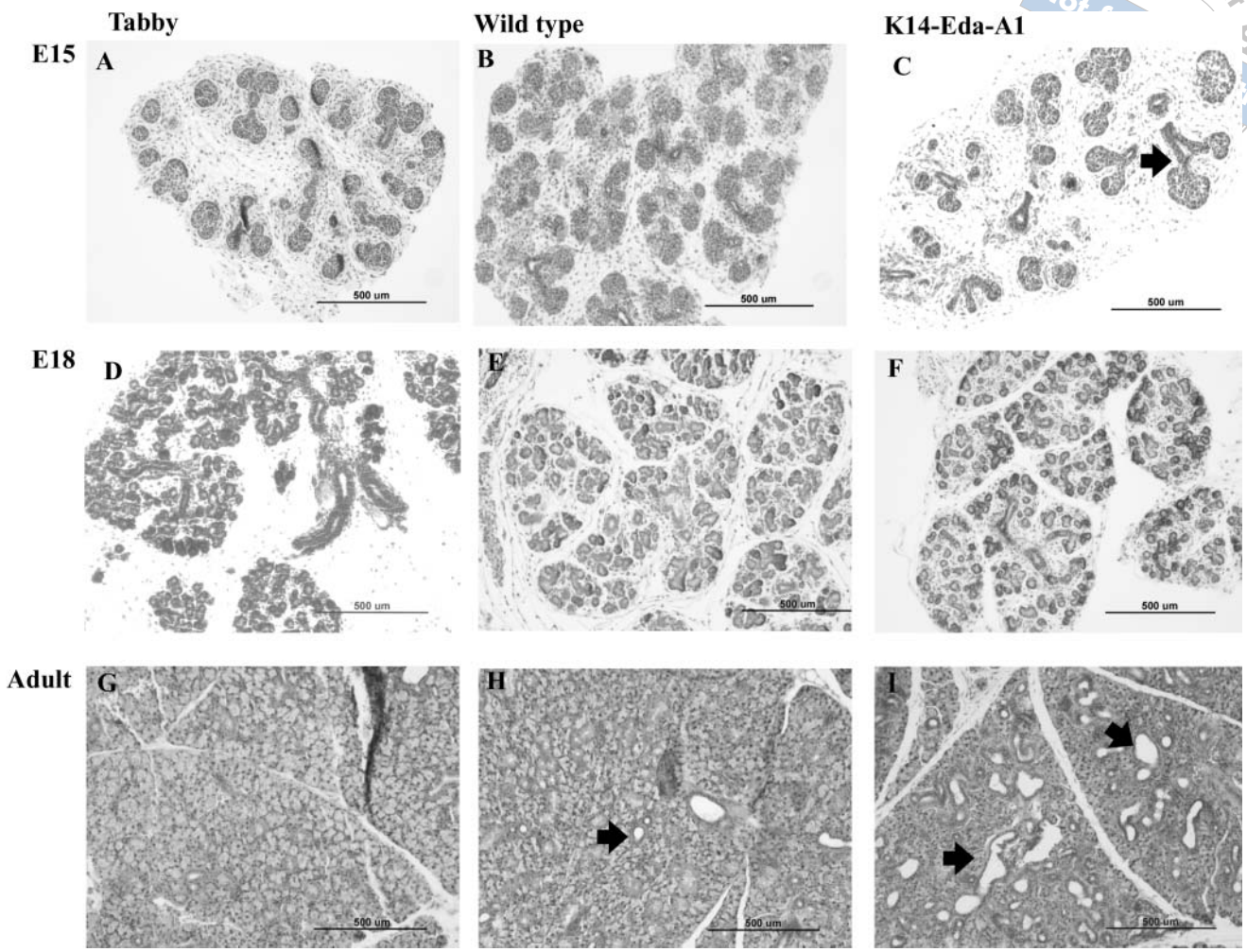

Fig. 1 Hematoxylin/eosin stained sections of Tabby, wild type and K14-Eda-A1 SMGs at E15 (A-C), E18 (D-F) and adult (G-I) developmental stages. The largest differences are seen in the adult SMGs. Tabby adult SMGs (G) demonstrated less ductal structures than wild type (H, arrow). The adult K14-Eda-A1 SMGs demonstrated a dysplastic glandular structure, with very large and abundant lumens as compared to wild type (I, arrows). At E15 the developing K14-Eda-A1 SMGs already show larger luminal structures than Tabby and wild type SMGs (A-C, arrow). At E18, however, the differences between the glands were less clear. Note that these sections not are suitable for comparisons of size and branch number as the sections are not from the same depth of the glands.

Analysis of Eda and Edar Expression in SMGs

To understand where the Eda/Edar signalling occurs in the tissues examined here, the expression patterns of *Eda* and *Edar* were studied by *in situ* hybridisation. The endogenous *Eda* transcription was detected in the mesenchyme in the wild type and K14-*Eda-A1* transgenic SMGs, but K14-*Eda-A1* transgenic glands also had strong ectopic expression in the outermost epithelium lining the canalicular structures facing the basement membrane in both embryonic and adult SMGs

Table 2 Mean branch numbers (±std) and branch ratio (mean terminal bud number after 44 hours/initial bud number±std) of K14-Eda-A1 SMGs as compared to wild type SMGs

	E13+6 h	P	E13+20 h	P	E13+30 h	P	E13+44 h	P	Branch ratio	P
K14-Eda-A1 (n=12) Controls (n=10)	9.0±2.9 6.6±2.6	0.06	28.4±11.1 15.5±7.46	0.01	50.3±17.6 26.8±11.0	0.02	75.9±22.3 45.2±29.2	0.01	12.1±14.7 6.3±0.6	0.14

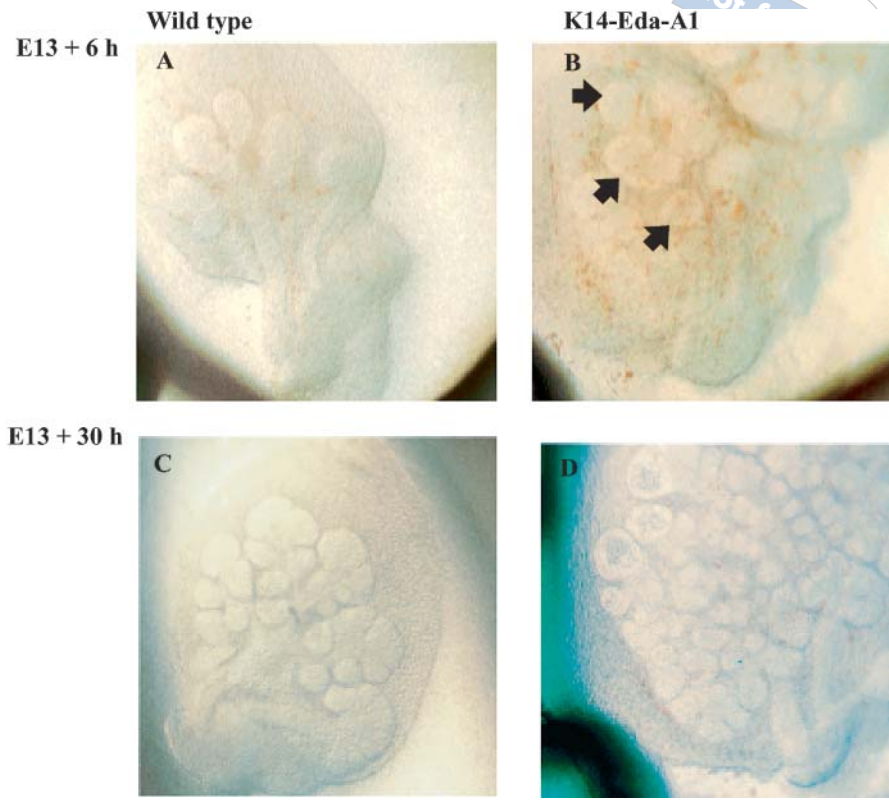

Fig. 2 SMGs from wild type (A, C) and K14-Eda-A1 (B, D) mice grown in culture. Already at E13+6 one can see slightly more epithelial branches in the transgenic glands as compared to controls (A, B, arrows) and the amount of new branches increased faster in the transgenic glands than in controls as exemplified in the photographs taken at E13+30 h (C, D). Original magnification: ×5.

(Figs. 3A-D). *Edar* transcription was seen in the outermost epithelium of E15 wild type and K14-*Eda-A1* transgenic SMG tissue (Figs. 3E, G). At E18 wild type and K14-*Eda-A1* SMGs demonstrated *Edar* expression mainly in the terminal end buds (data not shown). In adult SMGs *Edar* expression was also demonstrated in the epithelium, and no differences between wild type and K14-*Eda-A1* SMGs could be noted (Figs. F, H).

Analysis of other Transcripts with Developmental Functions in SMGs

Expression of *Tsc-22* in E15 and adult wild type, K14-*Eda-A1* and *Tabby* and E18 wild type and K14-*Eda-A1* SMGs is presented in Figure 4. *Tsc-22* was found to be weakly transcribed throughout wild type epithelial, and more strongly in wild type mesenchyme at E15 (Fig. 4B). *Tabby* mutant E15 SMG had *Tsc-22* expression in the mesenchyme only (Fig. 4A), whereas the K14-*Eda-A1* transgenic tissue sections showed *Tsc-22* expression also in epithelium at E15 (Fig. 4C). At E18, *Tsc-22* expression was mainly localised to the epithelium of the larger ductal structures in both wild type and K14-*Eda-A1* SMG tissue, but some expression was also seen in other epithelium (Figs. 4D, E). *Tsc-22* expression was seen in the in the luminal epithelium of adult wild type, K14-*Eda-A1* and *Tabby* SMGs, and this staining clearly demonstrated the difference in density of branches. K14-*Eda-A1* transgenic SMGs demonstrated strong expression of *Tsc-22* in very abundant epithelial structures as compared to wild type (Figs. 4G, H). Adult *Tabby* SMGs had fewer TSC-22 positive epithelial structures (Fig. 4F).

Expression of *Bmp4* mRNA in E15, E18 and adult wild type and K14-*Eda-A1* SMGs is shown in Figure 5 and it was detected in both epithelial and mesenchymal cells at all stages. Expression of *Fgf10, p21* and *Ptc* in E18 wild type and K14-*Eda-A1* SMGs is shown in Figure 6. Only weak, or absent, *Fgf10* transcription could be observed in all the examined E18 SMGs (Figs. 6A, B). *p21* expression was seen in the epithelium of the large ductal structures in both wild type and K14-*Eda-A1* transgenic E18 SMGs (Figs. 6C, D). *Ptc* was found to be transcribed in mesenchymal tissue, especially around the larger developing ductal structures in both E18 K14-*Eda-A1* and wild type SMGs (Figs. 6E, F).

DISCUSSION

Eda-A1 has an Important Role in Human and Mouse Salivary Gland Development

Eda/Edar signalling has been shown to be important in ectodermal organ development. *Tabby* mutants without functional Eda lack sweat glands, have abnormal hair and hypodontia (Mikkola and Thesleff, 2003). Overexpression of *Eda-A1* in mouse ectoderm causes formation of ectopic nipples, ectopic teeth, and increased function of sweat glands (Mustonen et al, 2003). Eda/Edar signalling has been suggested to be involved in the very early events of ectodermal organ development. In E13 skin *Eda* and *Edar* are coexpressed throughout the epithelium, but in E14 skin *Edar* expression becomes localized to the forming hair and tooth placodes, while *Eda* expression is downregulated in the placodal area and continues to be expressed in the interplacodal epithelium (Laurikkala et al, 2001, 2002).

The gene responsible for the phenotype of *Tabby* mice corresponds to the gene causing human XLHED. *Tabby* strains are therefore excellent models for the human condition. The present findings of reduced unstimulated and chewing stimulated whole salivary flow

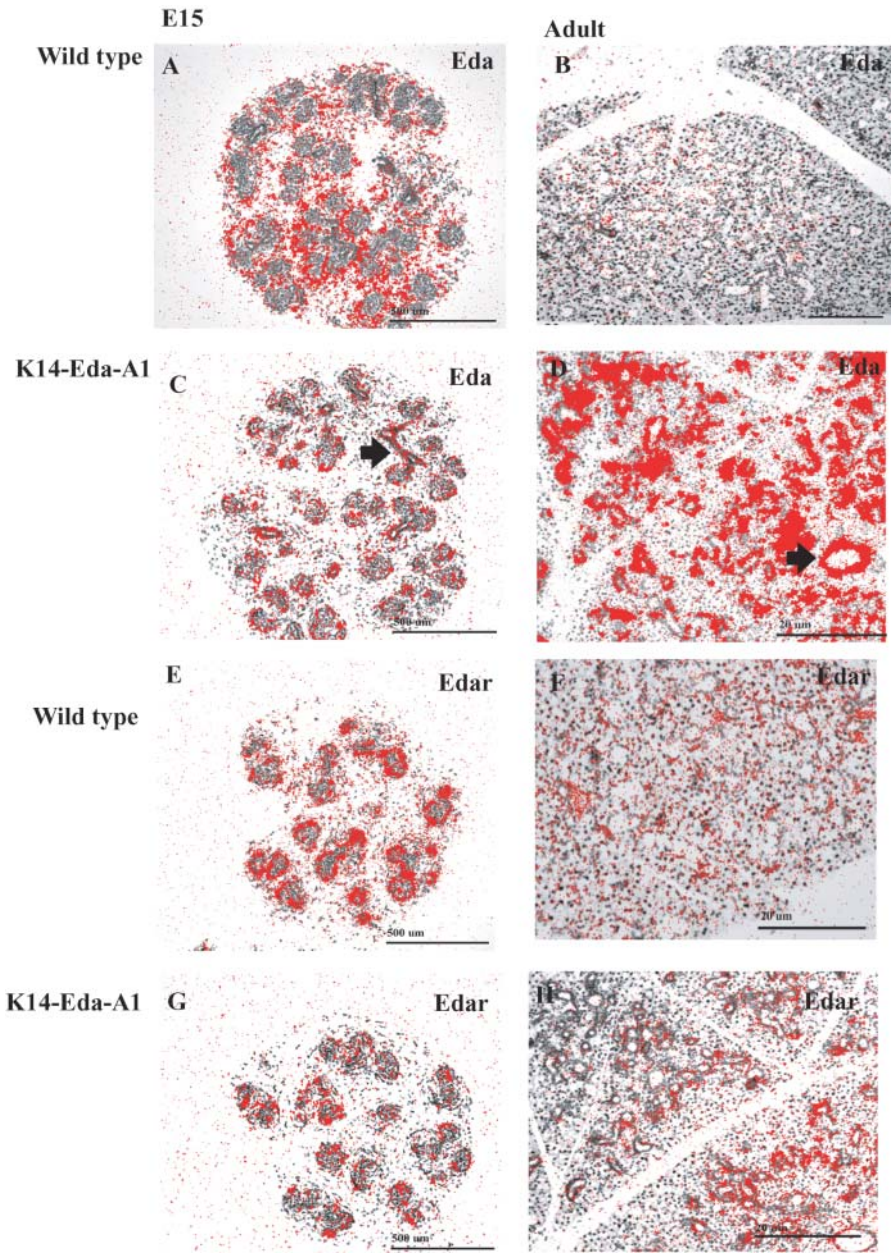

Fig. 3 Transcription of Eda and Edar in wild type and K14-Eda-A1 SMGs at E15 (A, C, E, G) and in adults (B, D, F, H). The endogenous Eda transcription was detected in the mesenchyme in the wild type and K14-Eda-A1 transgenic SMGs, but transgenic glands also had strong ectopic expression in the outermost epithelium lining the canalicular structures facing the basement membrane in E15 and adult SMGs (C, D, arrows). Edar transcription was seen in the outermost epithelium of E15 wild type and K14-Eda-A1 transgenic SMG tissue (E, G). In adult SMGs epithelial Edar signals was seen in both adult K14-Eda-A1 SMGs (F, H). Note that these sections not are suitable for comparison of size and branch number as the sections are not from the same depth of the glands.

as well as citric acid stimulated submandibular salivary flow in humans with XLHED, suggest that Eda-A1 plays a role in the development of human salivary glands. This is also confirmed in the previous report of hypoplasia and aplasia of major salivary glands in a person with XLHED (Nordgarden et al, 1998) and of fewer minor salivary glands and loose and disorganised structure of parotid gland tissue in a human foetus diagnosed with XLHED and aborted at 15 weeks of gestation (Nordgarden et al, 2001b). It has recently been shown that Edar signalling might be involved also in early formation of mouse SMGs, as *Edar* mRNA was detected in epithelial bud and branches at E12-E14 (Pispa et al, 2003). However, in an immunohistological study neither Eda nor Edar proteins could be detected in the developmental stages preceding the late pseudoglandular/early canalicular stage (Jaskoll et al, 2003), which may be due to lack of sensitivity of the

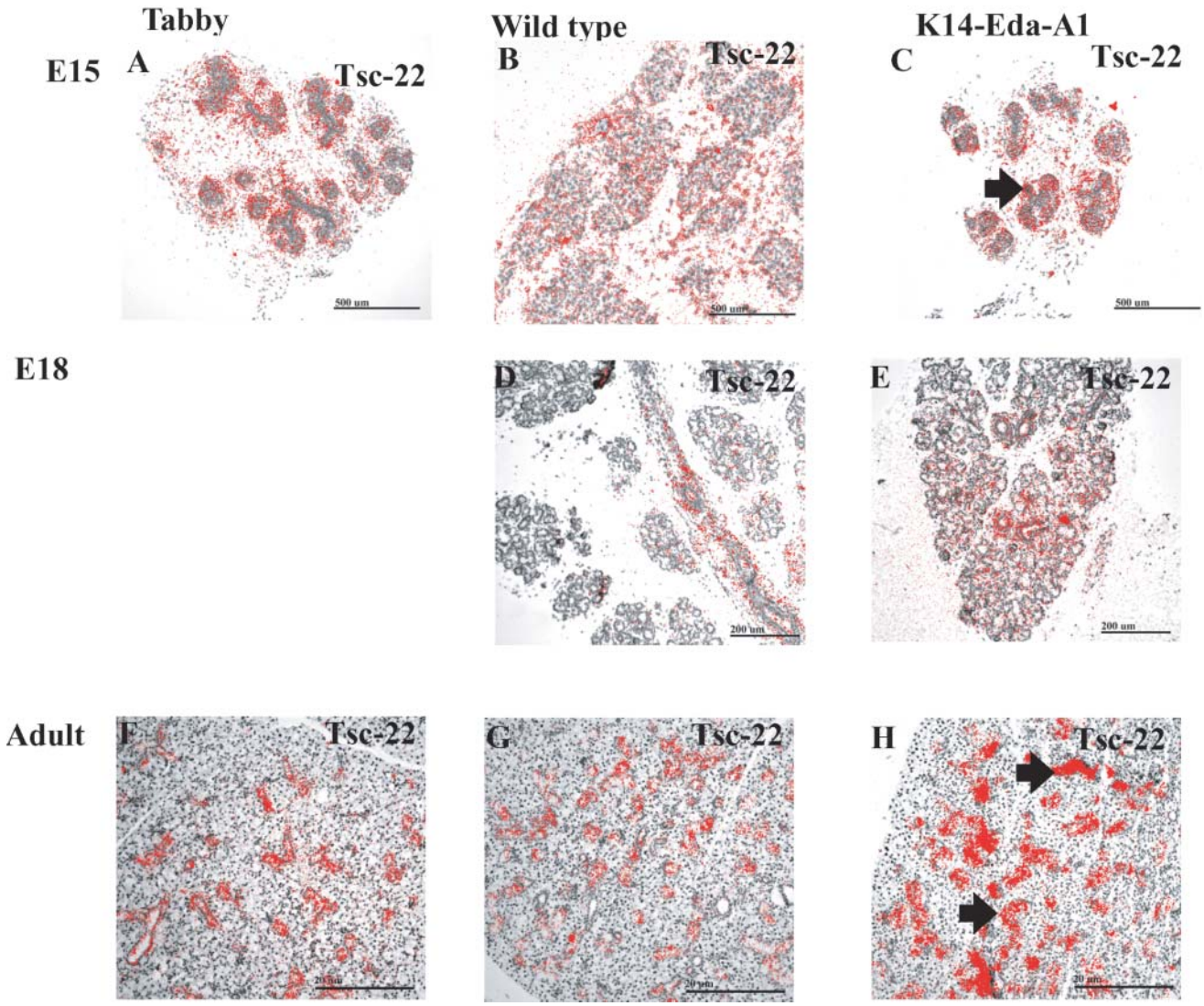

Fig. 4 Transcription of Tsc-22 in Tabby, wild type and K14-Eda-A1 SMGs at E15 (A-C) and adult (F-H) as well as in E18 wild type and K14-Eda-A1 SMGs (D, E). Tsc-22 was found to be weakly transcribed throughout wild type epithelial, and more highly in wild type mesenchyme at E15 (B). Tabby mutant E15 SMG had Tsc-22 expression in the mesenchyme only (A), whereas the K14-Eda-A1 transgenic tissue sections showed Tsc-22 expression also in epithelium at E15 (C, arrow). At E18, Tsc-22 expression was localised mainly to the epithelium of the larger ductal structures in both wild type and K14-Eda-A1 SMG tissue, but some expression was also seen in other epithelium (D, E). Both in wild type, K14-Eda-A1 and Tabby adult SMGs was Tsc-22 expression seen in the luminal epithelium, and this staining clearly demonstrated the difference in density of branches. K14-Eda-A1 transgenic SMGs demonstrated strong expression of Tsc-22 in very abundant epithelial structures (H, arrows), which seemed also to be larger than in wild type (G). Fewer such structures were seen in Tabby adult SMGs (F).

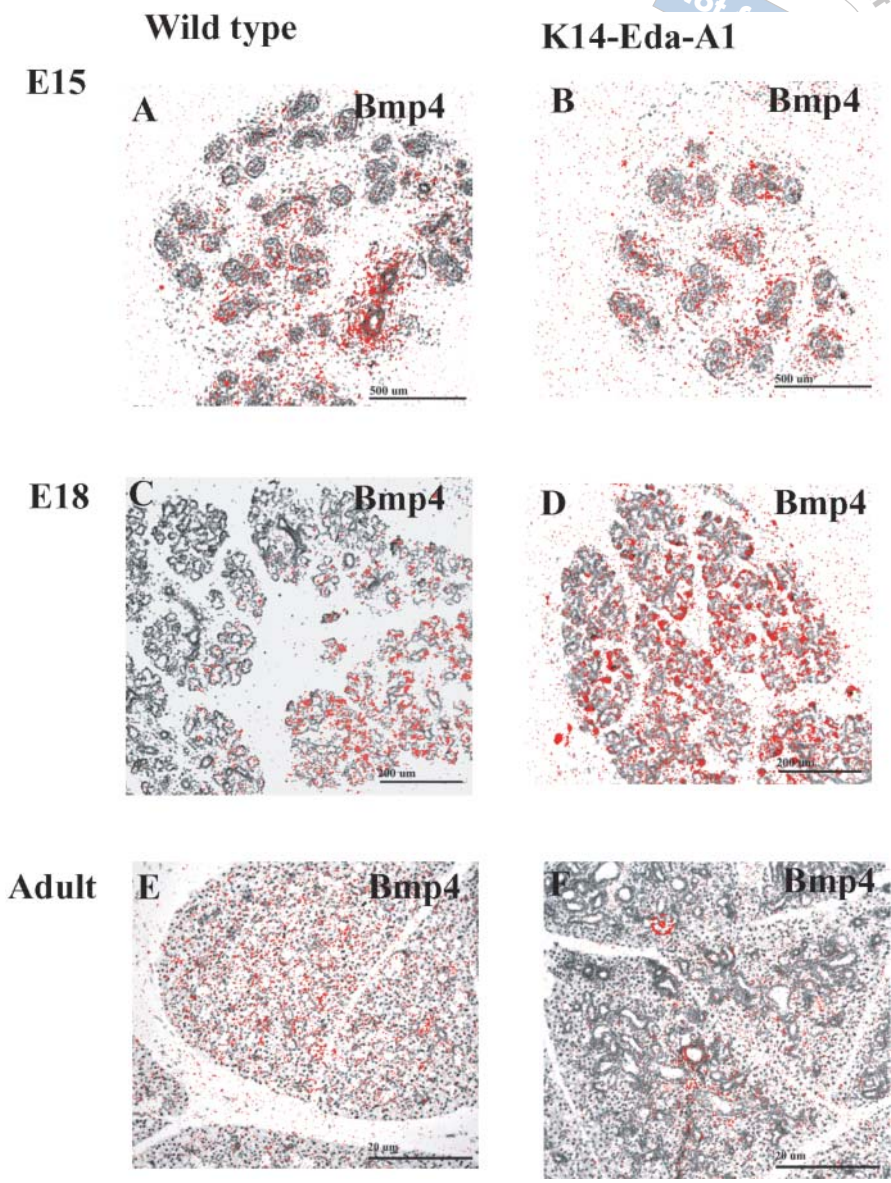

Fig. 5 Expression of Bmp4 mRNA in E15 (A, B), E18 (C, D) and adult (E, F) wild type and K14-Eda-A1 SMGs was detected in both epithelial and mesenchymal cells at all stages.

assay. Since increased branching in the K14-Eda-A1 SMGs cultured *in vitro* was demonstrated already at E13 in this study, the receptor protein must clearly be present at this time.

In the present study it is also shown that *Eda* is transcribed in wild type SMG mesenchyme and *Edar* in epithelial cells (Fig. 3), indicating a role in the epithelial-mesenchymal interactions and supporting a role in early branching events. The Eda protein has been immunolocalised to the epithelial surfaces at the sites of lumen formation (Jaskoll et al, 2003), indicating that efficient protein shedding from cell surfaces are important. It is also possible that the receptor bound protein is more stable and more concentrated at the sites of receptor expression.

Analysis of developing SMG in culture demonstrated that K14-*Eda-A1* transgenic SMGs overexpressing *Eda-A1* had more epithelial branches already at E13 (Table 2, Fig. 2). The phenotype in cultured SMGs with interrupted NF-κB signalling from E15 onwards (Melnick et al, 2001) demonstrated a less severe phenotype than *Tabby* SMGs, which lack Eda/Edar signalling also before E15. This indicates that Edar signalling is important during earlier stages of SMG development. Later, at E18, *Edar* was transcribed throughout the wild type epithelium of the SMGs, but a more intense signal was

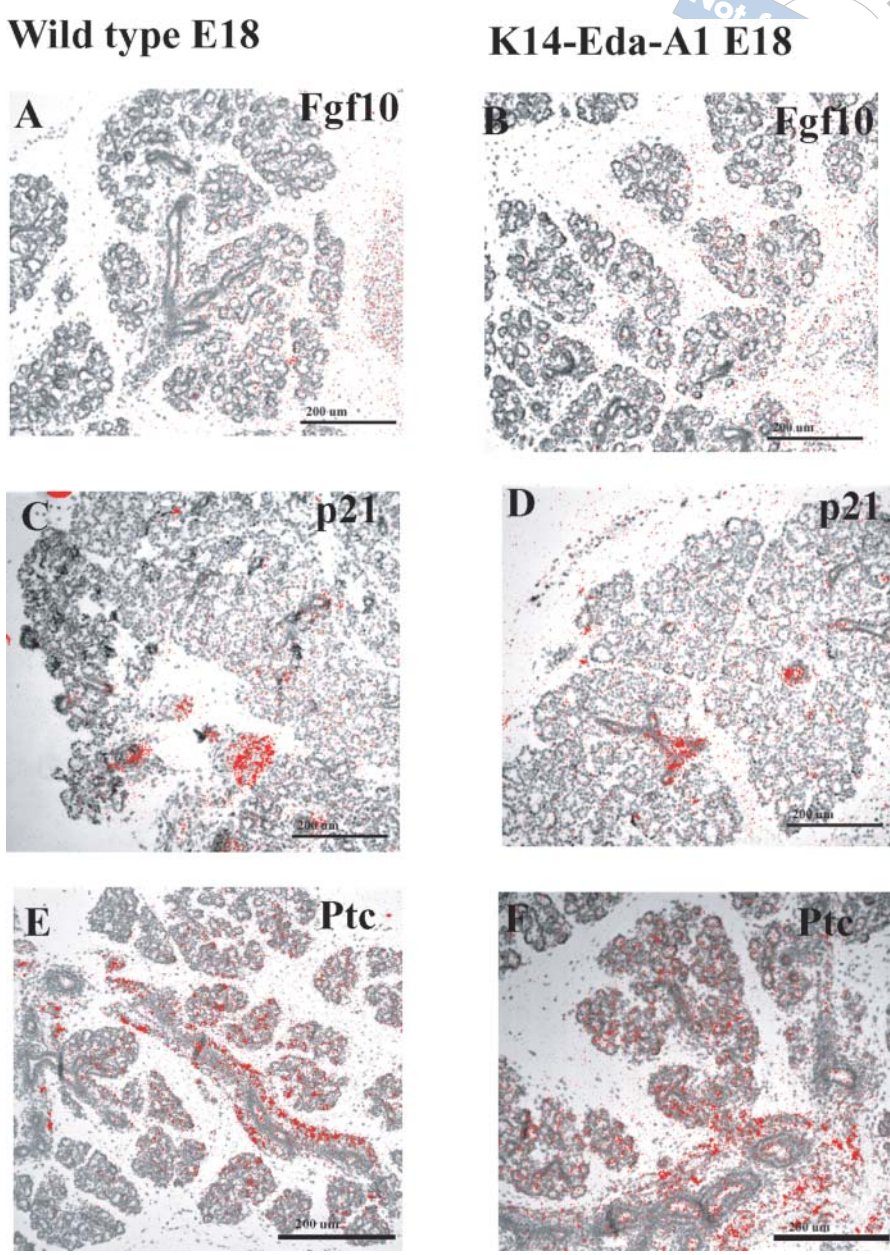

Fig. 6 Only weak, or absent, Fgf10 transcription could be observed in all the examined E18 SMGs A, B). p21 expression was seen in the epithelium of the large ductal structures in both E18 wild type and E18 K14-Eda-A1 transgenic SMGs (C, D). Ptc was found to be transcribed in mesenchymal tissue, especially around the larger developing ductal structures in both E18 K14-Eda-A1 and E18 wild type SMGs (E, F).

observed in the epithelium surrounding the larger lumina (data not shown). Hence it seems likely that the Eda/Edar signalling pathway has a role also in the formation of luminal structures in SMGs. The importance of Eda in branching events is indicated also by the fewer and smaller ductal structures in adult *Tabby* SMGs and the redundant and large lumens in K14-*Eda-A1* SMGs as compared to wild type SMGs (Figs. 1G-I).

K14 driven ectopic expression of *Eda* is extensive in the basal layer of the developing E15 and E18 epithelium (Fig. 3C, data not shown). Ectopic expression of *Eda-A1* did not seem to influence the transcriptional pattern of *Edar* at these stages (Fig. 3G, data not shown). This is in agreement with recent observations in K14-*Eda-A1* skin (Mustonen et al, 2003), where *Edar* was found to be upregulated in placodal epithelium and downregulated in interfollicular epithelium as in wild type mice (Headon and Overbeek, 1999; Laurikkala et al, 2001). However, K14-*Eda-A1* transgenic mice demonstrated developing hair follicles abnormally close to each other, indicating that Eda/Edar signalling negatively regulates lateral inhibition of placode formation in surrounding cells (Mustonen et al, 2003). Overexpression of *Eda-A1* in developing ectoderm resulted

in aberrant SMG development as larger luminal structures were observed in K14-*Eda-A1* E15 and E18 gland as compared to controls. Also, more branching was observed in the cultured E13 K14-*Eda-A1* SMGs than in wild type SMG cultures (Table 2, Fig. 2). Furthermore, supplementation of Eda-A1 to E14 SMG cultures resulted in a significant increase in branch number (Jaskoll et al, 2003). These findings indicate a similar mechanism of reduced lateral inhibition of branch formation of developing SMGs as in skin.

Adult K14-*Eda-A1* SMGs displayed abnormal ducts and blood vessels rather than acini, indicating that the effect of ectopic transcription of *Eda-A1 in vivo* has a greater effect on these structures. The last finding may be attributed to the activity of the K14 promoter in mouse SMGs (Wang et al, 1997), and thus a possible expression of *Eda* in the K14-*Eda-A1* transgenic ductal epithelium only.

Ectodysplasin Promotes the Formation of Tsc-22 Positive Epithelium

Tsc-22 is a transcription factor upregulated at many sites of epithelial-mesenchymal interactions during development. It was found as a clone stimulated by Tgf-β (Shibanuma et al, 1992), and it has been shown to be negatively regulated by BMPs and promoted by EGFs, Tgf-β and FGFs (Dohrmann et al, 2002). Based on observations in *Drosophila*, Tsc-22 family members are candidates for transcription factors that can integrate inputs to organize local signalling for formation of tissue boundaries (Dohrmann et al, 2002). In adult wild type SMG tissue, *Tsc-22* was expressed in ductal epithelium (Fig. 4G). This *Tsc-22* positive epithelium was reduced in *Tabby* SMGs (Fig. 4F), while K14-*Eda-A1* transgenic tissue had more abundant *Tsc-22* positive ductal epithelium (Fig. 4H). In wild type and *Tabby* E15 SMGs, *Tsc-22* was expressed in the mesenchyme, while in the K14-*Eda-A1* transgenic tissue expression was seen in both in the mesenchyme and the epithelial branches (Figs. 4A-C). In E18 tissue sections the expression was detected in epithelial and mesenchymal structures in both wild type and K14-*Eda-A1* transgenic SMGs (*Tabby* tissue not examined). The role of Tsc-22 at these sites is unknown, but it was associated with the luminal epithelium as ectopic *Eda-A1* increased the size of the *Tsc-22* positive epithelial branches.

The expression of other transcripts studied here, namely *Bmp4, Fgf10, p21* and *Ptc* did not differ between the K14-*Eda-A1* transgenic and wild type SMGs indicating that these molecules do not have a significant role in morphological changes observed in transgenic SMGs. However, it is possible that detectable differences could have been observed if SMGs had been examined at other time points of the developmental process, as changes in molecular signalling patterns are dynamic in all organ development. This is exemplified by the low or absent *Fgf10* transcription in both K14-*Eda-A1* and wild type SMGs at E18. *Fgf10* transcription has been found to peak at E13, at the time when branching morphogenesis starts to occur, and thereafter the expression declines (Hoffman et al, 2002).

In conclusion, Eda-A1 signalling seems to be important for branching morphogenesis and development of luminal structures in mouse SMGs. Based on the present and previous findings, it is reasonable to expect that Eda-A1 has a similar function in the development of human salivary glands.

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