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Defects and rescue of the minor salivary glands in Eda pathway mutants

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ABSTRACT

Despite their importance to oral health, the mechanisms of minor salivary gland (SG) development are largely unexplored. Here we present *in vivo* and *in vitro* analyses of developing minor SGs in wild type and mutant mice. *Eda*, *Shh* and *Fgf* signalling pathway genes are expressed in these glands from an early stage of development. Developing minor SGs are absent in Eda pathway mutant embryos, and these mice exhibit a dysplastic circumvallate papilla with disrupted *Shh* expression. Supplementation of Eda pathway mutant minor SG explants with recombinant EDA rescues minor SG induction. Supplementation with Fgf8 or Shh, previously reported targets of Eda signalling, leads to induction of gland like structures in a few cases, but these fail to develop into minor SGs.

Introduction

Salivary glands (SGs) have an important role in providing lubrication for digestion and protection of the oral tissues. In addition to the major SGs (reviewed in Tucker (2007)), the mammalian oral cavity also contains many minor SGs. These small glands provide ongoing protection to the oral tissues by secreting saliva continuously (Hand et al., 1999). Furthermore, study of developing minor SGs may reveal new information about the development of related epithelial structures such as the hair follicles, teeth or other glands. Despite their importance to oral health and potential interest to developmental biologists, the mechanisms of minor salivary gland (SG) development are largely unexplored.

The minor SGs are located in the submucosa of the tongue, cheeks and palate and secrete mostly mucus saliva. These glands are simpler in structure than the major SGs, lacking a complex branched ductal system and existing continuously within the surrounding connective tissue, rather than within a mesenchyme capsule (Hand et al., 1999). The development of the murine submandibular gland (SMG), the largest major SG, has been clearly characterized and develops from a thickening of the oral epithelium at E11.5, subsequently undergoing bud formation, branching morphogenesis and terminal differentiation. This process involves an array of signalling pathways including Sonic Hedgehog (Shh), Fibroblast Growth Factor (Fgf) and Ectodysplasin A (Eda) (Jaskoll and Melnick, 1999; Hoffman et al., 2002; Jaskoll et al., 2004a,b; Wells et al., 2010). By contrast, information on the genes involved in minor SG development is lacking, although it has been shown that the homeobox transcription factor *Nkx3.1* is expressed during mouse minor SG development and plays a role in duct morphogenesis (Schneider et al., 2000).

The Eda signalling pathway is required for ectodermal organ development. Mutations in *Eda* pathway genes cause hypohidrotic ectodermal dysplasia (HED), characterized by defective development of the hair, teeth and exocrine glands (Clarke et al., 1987). *EDA* encodes the tumour necrosis factor-like ligand EDA A1 (Srivastava et al., 1997). EDA A1 signals through its receptor EDAR and an intracellular adaptor protein EDARADD (Headon et al., 2001), and instigates downstream activation of NFκB (Kumar et al., 2001). Recessive mutations in *EDA* are responsible for X-linked HED, the most common form of HED (Kere et al., 1996), while dominant and recessive mutations in *EDAR* and *EDARADD* have been found in families carrying autosomal HED (Monreal et al., 1999; Headon et al., 2001). The spontaneous mouse mutants $Eda^{Ta/Ta}$, $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$ (Srivastava et al., 1997; Monreal et al., 1999; Headon et al., 2001) exhibit a similar phenotype to human HED in that the development of the hair, teeth and exocrine glands is impaired (Falconer et al., 1951; Grüneberg, 1965; Claxton, 1967; Grüneberg, 1971; Blecher et al., 1983). Analysis of Eda pathway mutant mice has revealed that Eda signalling plays a role in SMG branching morphogenesis (Jaskoll et al., 2003; Wells et al., 2010), but the role of Eda signalling in minor SG development has not been examined thoroughly. The classical study of gland development in $Eda^{Ta/Ta}$ states that lingual and palatal minor SGs are absent in $Eda^{Ta/Ta}$ embryos (Grüneberg, 1971), although it is unclear whether this represents developmental delay or a failure of initiation. As well as the minor SG defect, Grüneberg (1971) also reported that $Eda^{Ta/Ta}$ embryos exhibit a

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dysplastic circumvallate papilla (CVP), an epithelial structure at the back of the tongue associated with taste receptors and minor SGs.

Rescue of Eda pathway mutant phenotypes has been achieved using a number of methods. Injection of a recombinant form of EDA A1 given to pregnant $Eda^{Ta/Ta}$ mothers leads to rescue of the organs affected in the developing embryos (Gaide and Schneider, 2003), although the effect on the SGs was not documented. *In vitro*, addition of recombinant EDA A1 to $Eda^{Ta/Ta}$ skin or SMG cultures rescues primary hair placode and major SG branching respectively (Mustonen et al., 2004; Wells et al., 2010). Recent qPCR profiling has revealed downregulation of *Shh* and *Fgf8* in $Eda^{Ta/Ta}$ major SGs (Melnick et al., 2009). The branching defect in Eda pathway mutant SMG explants can be rescued with Shh, but not Fgf8 (Wells et al., 2010). *Shh* is also a target of Eda signalling in skin, but treatment of $Eda^{Ta/Ta}$ skin with exogenous Shh does not rescue primary hair placode formation (Pummila et al., 2007).

In this paper we have sought to provide the first characterization of minor SG development in wild type (WT) and Eda pathway mutant mice, and to test the ability of exogenous EDA A1, Fgf8 and Shh to rescue Eda pathway mutant minor SGs using a novel minor SG culture system.

Results

Eda pathway mutant embryos lack developing minor SGs

We began by analysing WT and Eda pathway mutant minor SG histology during mouse embryogenesis. In the WT, the glands present on the lateral part of the tongue are the first to initiate. These glands are first visible at E15.5 as downgrowths of the posterior lateral tongue epithelium. A number of lingual glands located closer to the midline initiate slightly later, and are visible at E15.5 as small buds (Fig. 1A, D). In $Eda^{Ta/Ta}$, $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$ embryos of the same stage, histological indications of developing minor SGs are absent (Fig. 1B–C, E. $Edaradd^{Cr/Cr}$ not shown).

In the E17.5 WT, some of the developing lingual glands have formed distinct ductal and acinar structures, with hollowed out ducts forming well defined lumina. There is evidence of mucus production at E17.5 and E18.5, as indicated by Alcian blue staining within the ducts and end buds. In addition, a number of glands are now visible on the palate (Fig. 1F,H,K). In Eda pathway mutant embryos of the same stage, histological signs of developing minor SGs are absent (Fig. 1G, I). The structures examined in the WT were confirmed to be developing minor SGs by *in situ* hybridisation of *Nkx3.1* (Schneider et al., 2000) (Fig. 1J).

The sequential appearance of minor SGs in different locations on the tongue and palate was consistent in all the embryos we examined, and we also noticed that glands simultaneously arose in identical locations on the left and right sides of the embryo (Fig. 1A). This indicates that the stage at which minor SGs develop, and their positioning within the oral cavity, is highly regulated.

Mucous minor SGs are absent in Eda pathway mutant adults

To confirm whether the embryonic Eda pathway mutant minor SG defects persist into adulthood, we compared sections of adult Eda pathway mutant and WT oral cavities (Fig. 2). In the WT, a large number of mucous glands are present on the palate and tongue. A number of serous glands are also observed in association with the CVP (Von Ebner's glands) anterior to the lingual mucous glands (Fig. 2A). In $Eda^{Ta/Ta}$, $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$, the tongue and palate were characterized by an absence of mucous glands. The associated von Ebner's glands (and other lingual serous glands) appeared normal (Fig. 2B and C. $Edaradd^{Cr/Cr}$ not shown). These data indicate that Eda signalling is required for the initiation of the mucous subset of palatal and lingual minor SGs.

$Eda^{Ta/Ta}$ *mice exhibit a dysplastic CVP with disrupted Shh expression*

While characterizing the minor SG phenotype in Eda pathway mutants, we also examined the CVP. In agreement with the observations of Grüneberg (1971), we confirmed that the embryonic CVP is dysplastic in $Eda^{Ta/Ta}$, $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$ (Fig. 3A and B. $Edar^{dlJ/dlJ}$ *and* $Edaradd^{Cr/Cr}$ not shown), appearing as a downward fissure rather than a raised dome. During WT early tongue development, *Shh* is expressed in the developing epithelial dome of the CVP where it is thought to regulate proliferation and epithelial invagination leading to the structure's characteristic morphology (Lee et al., 2006; Kim et al., 2009) (Fig. 3C). In $Eda^{Ta/Ta}$, *Shh* expression was restricted to one side of the developing CVP and was absent from the opposite wall of the dome where no epithelial trench had formed (Fig. 3D). Therefore, in the absence of *Eda*, CVP *Shh* expression appears disrupted, resulting in loss of the trench on one side.

Eda, Shh and Fgf pathway genes are expressed from the earliest stages of minor SG development

To shed light on the mechanisms regulating the induction and early development of the minor SGs, we studied the expression of a selection of genes during early minor SG development. Because minor SGs fail to initiate in Eda pathway mutant mice, we first chose to examine Eda pathway activity. In addition, we examined the expression of *Shh* and *Fgf* pathway genes, because these pathways are possible targets of Eda signalling (Pummila et al., 2007; Melnick et al., 2009). To study Eda pathway activity, we examined the expression of *Edaradd,* since the expression of this gene likely reflects the location where the pathway is acting. At E15.5, *Edaradd* was expressed in the budding minor SGs and throughout the oral epithelium (Fig. 3E and F). At the same stage, *Shh* mRNA was weakly detected in the budding glands but not throughout the oral epithelium (except in the developing rugae) (Fig. 3G and H). *Ptc1*, the Shh signalling receptor and target, was expressed in the budding glands, particularly at the distal portion of the bud (Fig. 3I). To examine Fgf signalling activity, we studied the expression of *Erm*, an Ets-family transcription factor target of Fgf signalling (Wasylyk et al., 1998). *Erm* was detected strongly in the budding glands at an early stage of development. *Erm* expression was confined to the developing glands, and was not detected in the oral epithelium (Fig. 3J).

Minor SGs fail to develop in $Eda^{Ta/Ta}$ *tongue explants*

We developed a novel minor SG culture system allowing us to follow the development of lingual minor SGs in tongue explants. After development of E13.5 WT whole tongues for 48 hours *in vitro*, developing minor SG buds were visible at the posterior lateral area of the tongue (Fig. 4A). Minor SG buds were absent in $Eda^{Ta/Ta}$ tongues at this stage (Fig. 4C). After 72 hours in culture, more buds were visible on the lateral WT tongue, and further buds appeared closer to the midline (Fig. 4B), in agreement with our histological data. Developing lingual glands remained absent in $Eda^{Ta/Ta}$ tongues at this stage (Fig. 4D).

To quantify these observations, the tongue explants were sectioned and subjected to histology staining and *in situ* hybridisation of an *Nkx3.1* probe. Developing minor SGs, as defined by *Nkx3.1* expressing epithelial thickenings, were counted over all sections from each explant, and the number of developing glands in WT, $Eda^{Ta/+}$ and $Eda^{Ta/Ta}$ explants compared. While no *Nkx3.1* expressing regions were present in $Eda^{Ta/Ta}$ tongues (Fig. 4F, H, I), a mean of ~6 *Nkx3.1* positive developing lingual glands were present in WT tongues (Fig. 4E, G, I). *Nkx3.1* positive lingual glands were present in $Eda^{Ta/+}$, but appeared reduced in number in comparison with WTs (Fig. 4I).

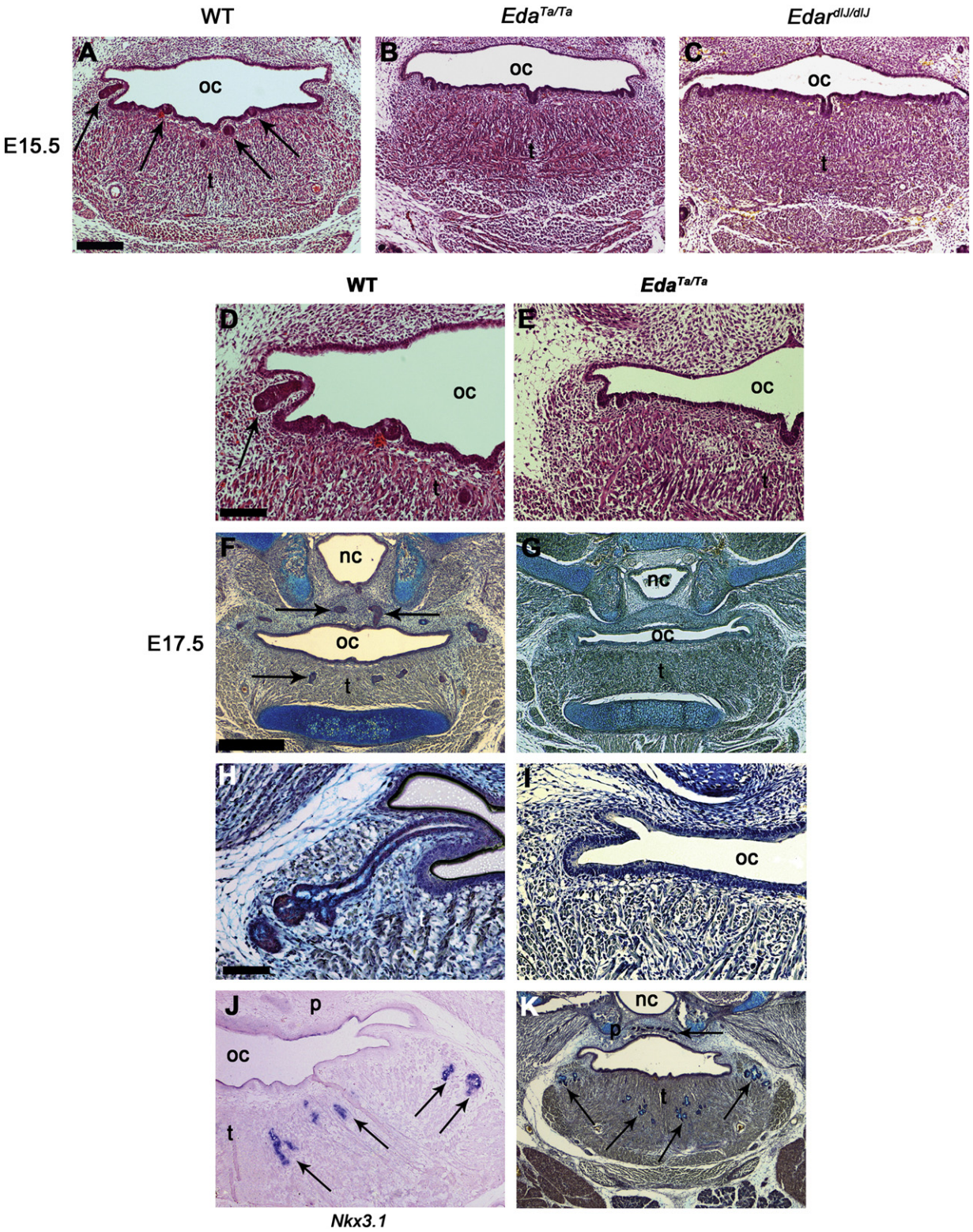


Fig. 1. Minor SGs are absent in Eda pathway mutant embryos. Frontal sections. (A) E15.5 WT oral cavity showing epithelial buds and downgrowths (arrows). (B) E15.5 *Eda*$^{Ta/Ta}$ ($n=6$) oral cavity stage matched to (A). Developing minor SGs are absent. (C) E15.5 *Edar*$^{dlJ/dlJ}$ ($n=5$) oral cavity stage matched to (A) showing similar absence of developing minor SGs. (D) High magnification of E15.5 WT oral cavity showing downgrowth of the posterior lateral tongue epithelium (arrow), and additional buds towards the midline. (E) High magnification of *Eda*$^{Ta/Ta}$ tongue epithelium. Developing minor SGs are absent. (F) E17.5 WT oral cavity. A number of developing lingual and palatal minor SGs are seen (arrows). (G) E17.5 *Eda*$^{Ta/Ta}$ ($n=3$) oral cavity stage matched to (F). Developing minor SGs remain absent. (H) High magnification of E17.5 WT lingual gland. Distinct ductal and acinar structures have formed, and the duct exhibits a well-defined lumen. Alcian blue staining indicates mucus production. (I) High magnification of E17.5 *Eda*$^{Ta/Ta}$ oral cavity showing absence of developing minor SGs. (J) E17.5 oral cavity. *Nkx3.1* is expressed in developing minor SGs (arrows). (K) WT E18.5 oral cavity. Alcian blue staining indicates differentiated glands on the tongue and a number of palatine minor SGs are present (arrows). nc, nasal cavity; oc, oral cavity; t, tongue; p, palate. Scale bar in A (for A–C, J) = 200 μm. Scale bar in D (for D, E) = 100 μm. Scale bar in F (for F, G, K) = 500 μm. Scale bar in H (for H, I) = 100 μm.

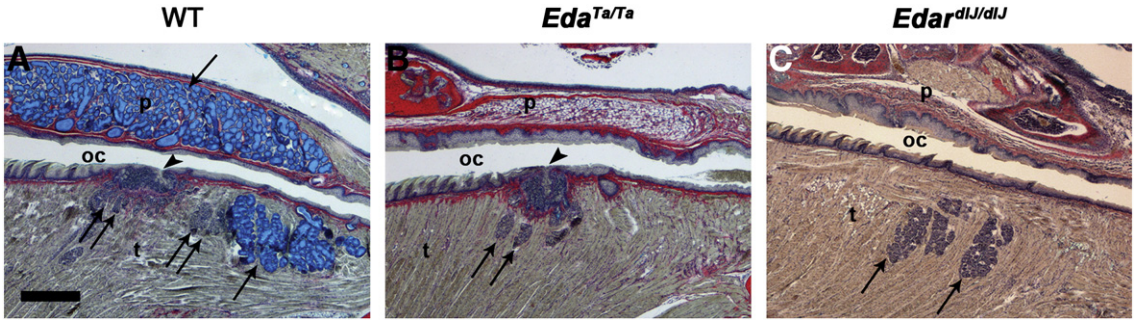


Fig. 2. Mucous minor SGs are absent in Eda pathway mutant adults. Trichrome stained sagittal sections of the oral cavity in 6-week-old mice. (A) WT FVB/N tongue and palate showing Alcian blue stained mucous minor SGs (arrows) and unstained lingual serous minor SGs (double arrows). CVP indicated by arrowhead. (B) *Eda*$^{Ta/Ta}$ ($n=3$) tongue and palate. Mucous minor SGs are absent, but lingual serous SGs are present (arrows). (C) *Edar*$^{dlJ/dlJ}$ ($n=3$) tongue and palate. Mucous minor SGs are absent as in (B). t, tongue; oc, oral cavity; p, palate. Scale bar in A (for A–C) = 500 μm.

Exogenous EDA A1 rescues minor gland induction in Eda$^{Ta/Ta}$

We investigated whether minor SG induction can be rescued by recombinant EDA A1 supplementation *in vitro*. E13.5 *Eda*$^{Ta/Ta}$ and *Eda*$^{Ta/+}$ tongues were cultured with 500 ng/mL EDA A1, and control tongues were cultured in the absence of EDA A1. After 4 days in culture, tongue explants were sectioned and subjected to histology staining and *in situ* hybridisation of an *Nkx3.1* probe. Developing minor SGs, as defined by *Nkx3.1* expressing epithelial thickenings, were counted over all sections from each explant, and the mean number of developing glands in treated and control explants compared. While no *Nkx3.1* expressing regions were present in control *Eda*$^{Ta/Ta}$ tongues (Fig. 5A, C, E), a mean of ~4 *Nkx3.1* positive developing lingual glands were present in EDA A1 treated *Eda*$^{Ta/Ta}$ tongues (Fig. 5B, D, E). A trend towards increased minor gland number was also observed in WT and *Eda*$^{Ta/+}$ tongues treated with EDA A1 in comparison with untreated WT tongues (Fig. 5E).

Exogenous Shh and Fgf8 do not fully rescue minor gland induction in Edar$^{dlJ/dlJ}$

We tested whether application of exogenous Shh or Fgf8, potential targets of Eda signalling, would result in rescue of the minor SGs in Eda pathway mutant tongues. For these experiments, we moved to *Edar*$^{dlJ/dlJ}$ tongues. These mice lack functional *Edar*, and we considered them the best system for a downstream rescue since the possibility of activating signalling via *Edar* is abolished. E13.5 *Edar*$^{dlJ/dlJ}$ tongues were cultured with either 500 ng/mL Fgf8b or 2.5 μg/mL Shh-N peptide, and control tongues were cultured in the absence of recombinant protein. These proteins were also tested simultaneously on SMG explants to confirm that they were functional (Wells et al., 2010). After 4 days in culture, tongue explants were sectioned and subjected to histology staining and *in situ* hybridisation of an *Nkx3.1* probe. No *Nkx3.1* expression was detected in control *Edar*$^{dlJ/dlJ}$ explants. In 3/19 Fgf8b-treated tongues, weak *Nkx3.1* expression was evident in 1 or 2 gland-like structures, indicating possible rescue of minor SGs in a limited number of explants (Fig. 6A–D). No *Nkx3.1* expression was detected in Shh-treated *Edar*$^{dlJ/dlJ}$ explants, although *Nkx3.1*-negative gland like structures were noted. In location and morphology, these structures were similar to minor SGs, suggesting that Shh causes the induction of a small number of glands which then do not develop further (Fig. 6E–H, J). Expression of *Nkx3.1* was detected in developing glands in *Edar*$^{dlJ/+}$ tongues as expected (Fig. 6I).

Discussion

Minor SGs develop by epithelial downgrowth and are highly regulated

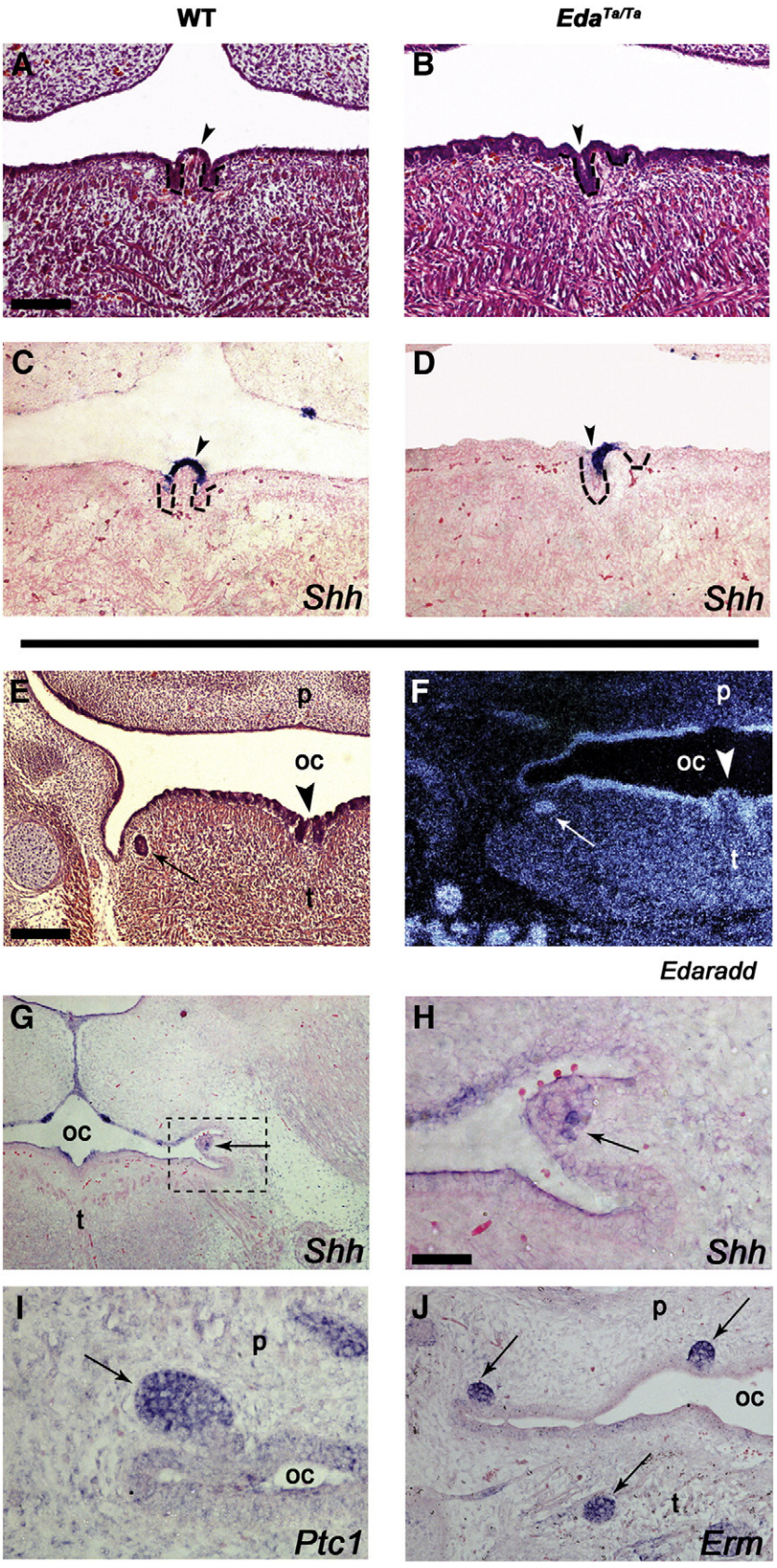
Our data represents, to our knowledge, the first thorough description of minor SG development in the mouse. We show that the minor SGs develop by epithelial thickening and budding, and subsequent downgrowth and differentiation. However, unlike the SMGs, the minor SGs lack a mesenchyme capsule (Hand et al., 1999). This raises the question of where the signals instructing the positioning and development of the glands reside. Recombination experiments have shown that major SG branching is driven by the surrounding mesenchyme (Grobstein, 1953), but it has been speculated that the initiation signals may come from the oral epithelium as in the developing tooth (Tucker, 2007). Our data showing that the minor glands are able to initiate development without signals from a mesenchyme capsule certainly suggests that the major SGs may be able to do the same, only requiring mesenchymal signals to instruct later branching morphogenesis. It is tempting to speculate that the minor SGs do not branch extensively because they do not have access to such stimulatory signals as those secreted by the major SG mesenchyme.

To date the only known marker of developing minor SGs was *Nkx3.1* (Schneider et al., 2000). Here we show that *Edaradd*, *Shh, Ptc1* and *Erm* also mark the minor SGs from the earliest stages of their development. Shh is a known mitogen (Ingham and McMahon, 2001) and it seems likely that its function in the developing minor SG is to promote proliferation and epithelial downgrowth, as in the developing hair follicle (Millar, 2002). In addition, our data showing expression of both *Ptc1* and *Shh* in the epithelium, rather than in separate tissue compartments, indicates that Shh signalling regulates minor SG development in an autocrine manner. Fgf signalling is required for major SG development (reviewed in Tucker (2007)), and our finding of strong *Erm* expression in the developing minor SGs is in agreement with this.

Fig. 3. Dysplastic CVP in *Eda*$^{Ta/Ta}$ and minor SG gene expression. Frontal sections. (A) E15.5 WT oral cavity, showing developing CVP (arrowhead) with trenches outlined. (B) E15.5 *Eda*$^{Ta/Ta}$ oral cavity stage matched to (A) The CVP is dysplastic (arrowhead) and the trenches either side of the dome are uneven in depth (outlined). (C) Serial section to (A). *Shh* is expressed in the developing CVP dome (arrowhead). Expression is absent from the trenches (outlined). (D) Serial section to (B) showing restriction of *Shh* expression to one side of developing *Eda*$^{Ta/Ta}$ CVP. (E) H&E stained E15.5 oral cavity showing developing minor SG (arrow). (F) E15.5 ^{35}S *in situ* hybridisation. *Edaradd* is expressed in the oral epithelium and minor SG (arrow). (G) *In situ* hybridisation of E15.5 oral cavity showing *Shh* expression in developing minor SG (arrow) and palatal rugae. (H) High power of dashed box in (G). (I) E16.5 *In situ* hybridisation. *Ptc1* is expressed in developing minor SG (arrow), especially at the distal portion of the bud. (J) E16.5 *in situ* hybridisation. *Erm* is expressed in the developing minor SGs (arrows). oc, oral cavity; t, tongue; p, palate. Scale bar in A (for A–D, J) = 100 μm. Scale bar in E (for E–G) = 200 μm. Scale bar in H (for H–I) = 50 μm.

Eda signalling is required for minor SG initiation

Our data showing Eda pathway gene expression in the developing minor SGs and our analyses of minor SG development in Eda pathway mutant mice indicate a requirement for Eda signalling in minor SG initiation. The observation that $Eda^{Ta/+}$ embryos exhibit a mild phenotype with respect to their minor SGs indicates that levels of Eda signalling are important in minor SG development. This idea fits with



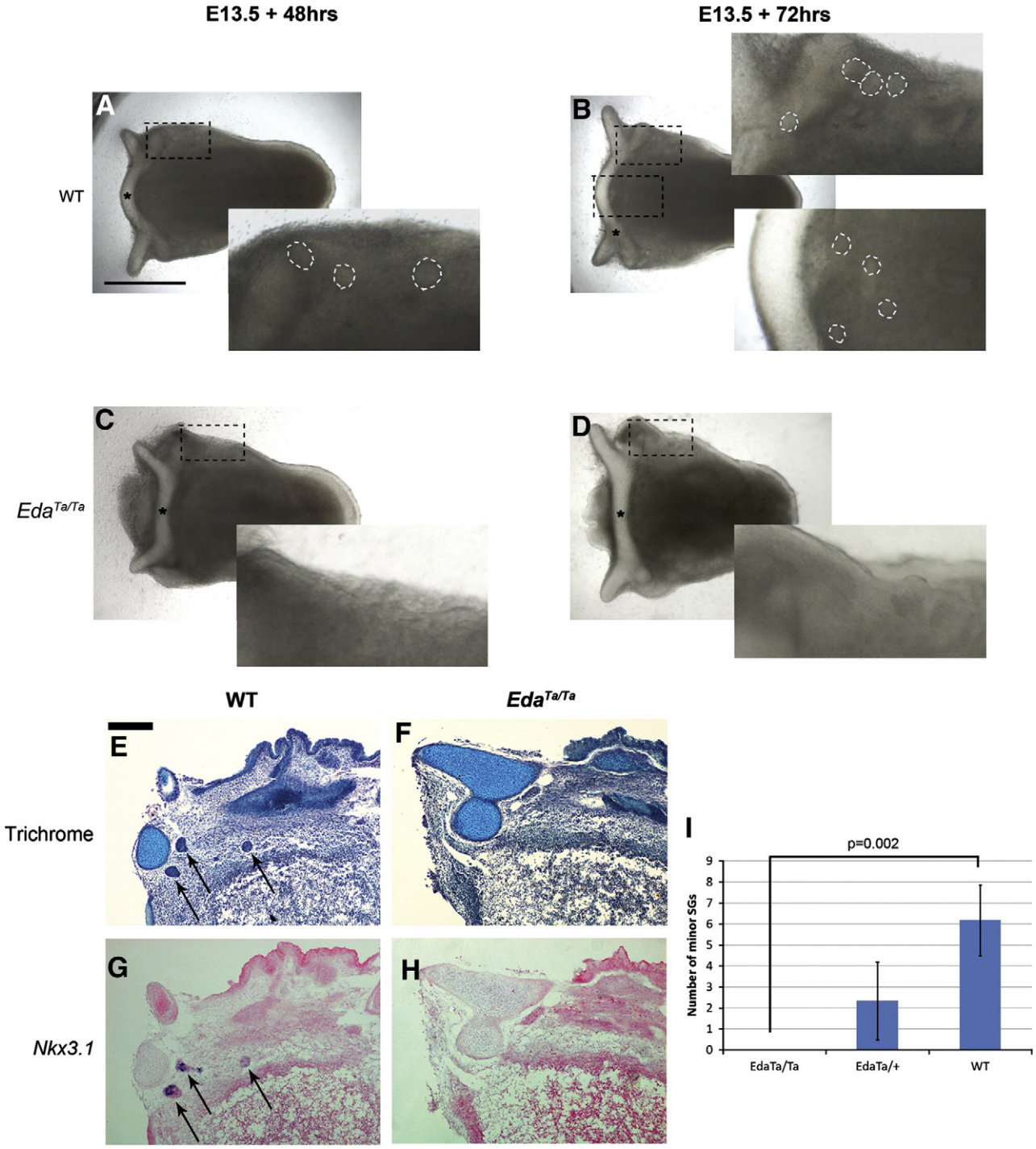


Fig. 4. Minor SGs fail to develop in *Eda*$^{Ta/Ta}$ tongue explants. Tongue explants in culture (A–D) and sectioned (E–H). Insets in A–D show high magnification of dashed boxes. (A) E13.5 WT tongue explants after 48 hrs in culture. Lingual epithelial buds are visible (outlined). (B) Explant shown in (A) after 72 h in culture. More epithelial buds are visible on lateral tongue and towards the midline (outlined). (C) E13.5 *Eda*$^{Ta/Ta}$ tongue explant after 48 h in culture. Budding minor SGs are absent. (D) Explant shown in (C) after 72 hrs in culture. Developing minor SGs remain absent. (E) E13.5 + 72 h WT posterior tongue section (trichrome stained). Developing lingual glands are apparent (arrows). (F) E13.5 + 72 hrs *Eda*$^{Ta/Ta}$ posterior tongue section. Developing lingual glands are absent. (G) Serial section to (E), showing *Nkx3.1* expression in developing lingual glands (arrows). (H) Serial section to (F). *Nkx3.1* expression is absent. (I) Chart representing mean lingual gland number counted per genotype group. Number of explants per group: *Eda*^{Ta} ($n=7$), *Eda*$^{Ta/+}$ ($n=3$), WT ($n=6$). Error bars represent one standard error ± mean. Scale bar in A (for A–D) = 1000 μm. Scale bar in E (for E–H) = 200 μm. * indicates hyoid cartilage.

data showing that human female carriers of XL-HED exhibit reduced saliva flow (Lexner et al., 2007). No information is yet available on the signalling mechanisms involved in initiating development of major or minor SGs; our work indicates an involvement for Eda signalling. The literature also lacks information on genes involved in determining the type of SG that will develop. The specific requirement for Eda in mucous minor SG initiation contrasts with the case of the SMGs (Melnick et al., 2009) and serous minor SGs, which do not require Eda signalling for initiation, and evokes the different requirements for Eda in the distinct types of hair follicles. In *Eda*$^{Ta/Ta}$, secondary and tertiary hair follicles initiate normally, but primary hair follicles fail to initiate (Laurikkala et al., 2002).

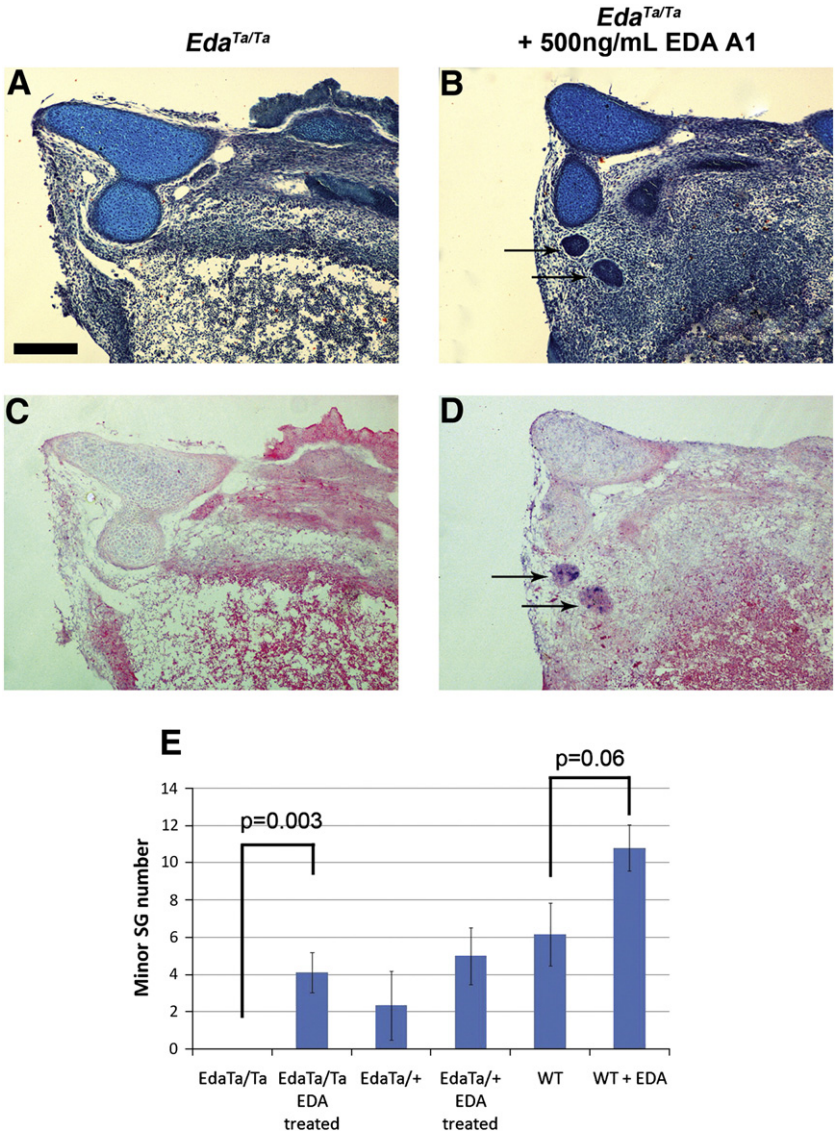


Fig. 5. Exogenous EDA A1 rescues lingual gland development in $Eda^{Ta/Ta}$. Trichrome stained sections of E13.5 tongue explants cultured for 4 days (A–B), and serial sections subjected to *Nkx3.1 in situ* hybridisation (C–D). (A) $Eda^{Ta/Ta}$ posterior tongue showing lack of developing lingual glands and (B) *Nkx3.1* expression. (C) $Eda^{Ta/Ta}$ posterior tongue treated with 500 ng/mL EDA A1 *in vitro*. Rescued lingual glands are apparent (arrows). (D) Serial section to (C). *Nkx3.1* is expressed in rescued lingual glands (arrows). (E) Chart representing mean lingual gland number counted per genotype and treatment group. Number of explants per group: $Eda^{Ta/Ta}$ ($n=7$), $Eda^{Ta/Ta}$ + EDA ($n=8$), $Eda^{Ta/+}$ ($n=3$), $Eda^{Ta/+}$ + EDA ($n=3$), WT ($n=6$), WT + EDA = ($n=5$). Error bars represent one standard error ± mean. Scale bar in A (for A–D) = 200 μm.

Eda pathway mutant minor SGs can be rescued with recombinant EDA A1 in vitro

Using our minor SG culture system, we show that supplementation of E13.5 $Eda^{Ta/Ta}$ tongues with EDA A1 results in rescue of gland development. This agrees with data showing that exogenous EDA A1 rescues primary hair follicle development in $Eda^{Ta/Ta}$ skin explants (Laurikkala et al., 2002). EDA A1 injection has been shown to rescue most aspects of the $Eda^{Ta/Ta}$ phenotype (Gaide and Schneider, 2003) and as a result recombinant EDA A1 is promising a cure for sufferers of HED. HED patients demonstrate reduced whole saliva flow (Nordgarden et al., 2001) leading to xerostomia (dry mouth), a reduced quality of life and further damage to an already compromised dentition. However, the ability of this protein to rescue minor SG development in $Eda^{Ta/Ta}$ had not been studied. Our findings are encouraging for future attempts to correct SG defects in HED patients. Although our histological studies showed an absence of minor SGs in Eda pathway mutant embryos, the fact that these glands are rescued with EDA A1, and that they develop in the expected position, implies that a group of cells able to respond to the signal are in fact present in Eda pathway mutants.

Exogenous Shh and Fgf8 do not fully rescue minor gland induction in $Edar^{dlJ/dlJ}$

QPCR data has indicated that *Shh* is a potential target of Eda signalling in the SMGs (Melnick et al., 2009), and treatment of embryonic $Eda^{Ta/Ta}$ skin cultures with recombinant EDA A1 results in upregulation of *Shh* (Pummila et al., 2007). A number of *Nkx3.1* expression domains in the developing embryo, such as the prostate gland, are dependent on *Shh* (Schneider et al., 2000). We were unable to demonstrate rescue of *Nkx3.1* expression in $Edar^{dlJ/dlJ}$ tongue explants by Shh supplementation, although we did observe the presence of gland-like tissue. *Shh* overexpression is associated with tumour formation in the skin (Oro et al., 1997), and it is possible that the tissue we observe is the result of overproliferation. Our finding that Shh is not sufficient to completely rescue minor SG development in the absence of Eda signalling agrees with data showing that exogenous Shh is unable to rescue primary hair placode induction in $Eda^{Ta/Ta}$ skin cultures (Pummila et al., 2007).

Fgf8 is another potential target of Eda signalling in the SMGs (Melnick et al., 2009). Other studies have shown growth factor mediated rescue of aspects of the $Eda^{Ta/Ta}$ phenotype. EGF injection

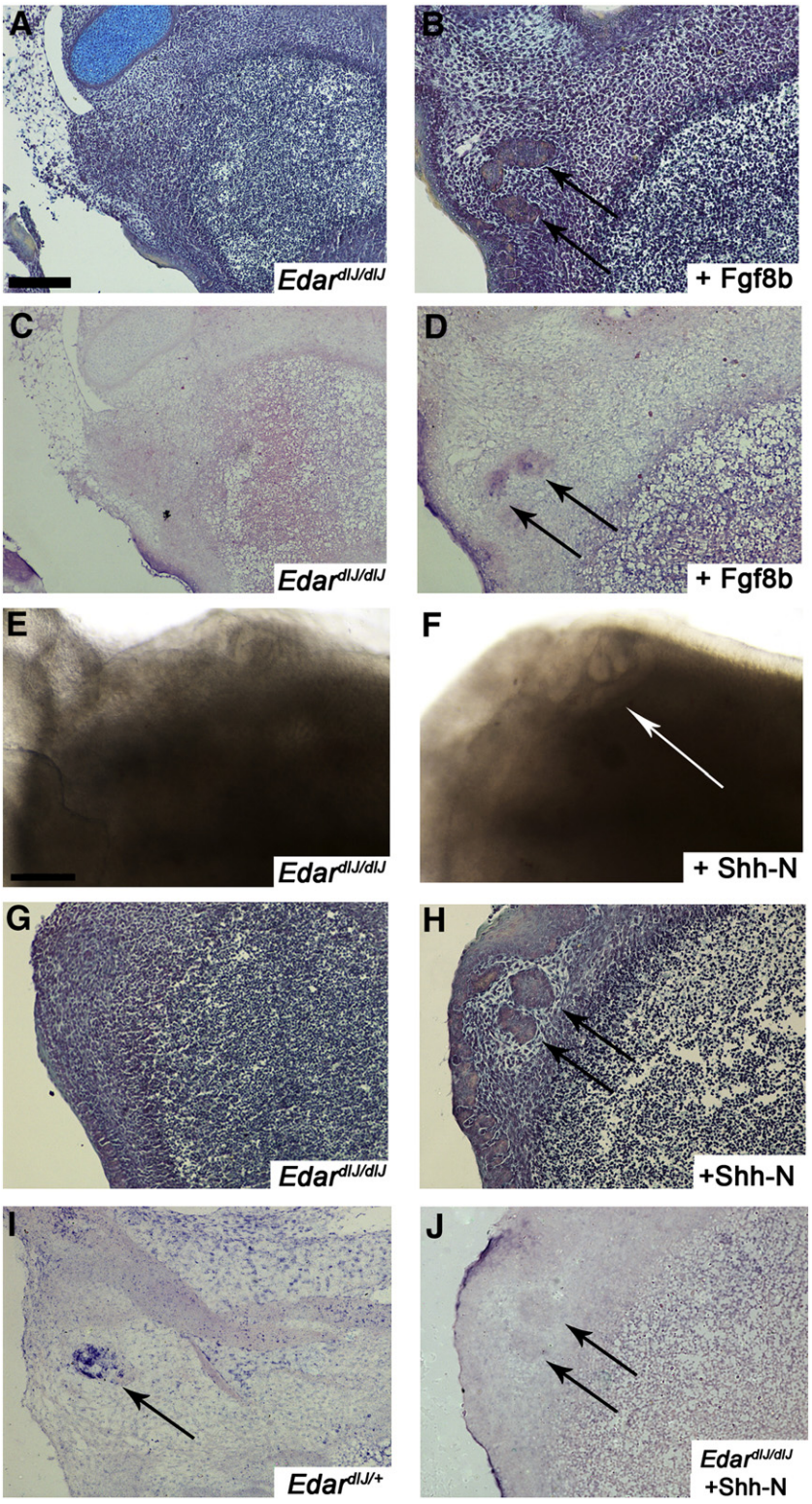


Fig. 6. Exogenous Fgf8 and Shh do not fully rescue lingual gland development in $Edar^{dlJ/dlJ}$. E13.5 tongue explants after 4 days in culture. (A) Trichrome stained section of $Edar^{dlJ/dlJ}$ posterior tongue showing lack of developing lingual glands and (C) serial section showing lack of *Nkx3.1* expression. (B) Trichrome stained section of $Edar^{dlJ/dlJ}$ tongue treated with 500 ng/mL Fgf8b. Gland-like structures are evident (arrows). (D) Serial section to (B). *Nkx3.1* is weakly expressed in these structures (arrows). (E) $Edar^{dlJ/dlJ}$ control tongue explant. (F) $Edar^{dlJ/dlJ}$ tongue explant treated with 2.5 μg/mL Shh-N. Rescued gland-like structures are evident (arrow). (G) Trichrome stained section of control $Edar^{dlJ/dlJ}$ tongue. (H) Trichrome stained section of $Edar^{dlJ/dlJ}$ tongue treated with 2.5 μg/mL Shh-N showing gland-like structures (arrows). (I) Control $Edar^{dlJ/+}$ tongue section. *Nkx3.1* expression is detected in developing lingual gland (arrow). (J) Serial section to (H). *Nkx3.1* is not expressed in the gland-like structures. Scale bar in A (for A–D, G–J) = 100 μm. Scale bar in E (for E–F) = 200 μm.

administered to $Eda^{Ta/Ta}$ pups rescues sweat gland development (Blecher et al., 1990). In embryonic $Eda^{Ta/Ta}$ tooth explants, cusp morphogenesis is partially rescued by exogenous Fgf10 (Pispa et al., 1999), although it is unclear whether this is through a mechanism independent of Eda signalling, such as an increase in epithelial proliferation. In our study, 3 out of 19 cultures showed induction of *Nkx3.1*-positive minor SG structures in response to Fgf8 supplementation, indicating that Fgf8 is not sufficient to rescue reliably. This may

be due to the greater importance of alternative Fgf ligands in minor SG development, or a subtle difference in the developmental stage of the tongue explants which did show a rescue. It seems plausible that neither Fgf8 nor Shh are able to act alone in minor SG development and may require other factors for normal morphogenesis, or act in concert with each other.

Materials and methods

Animals

All Eda pathway mutant mice were on the FVB/N background. E13.5 WT, $Eda^{Ta/+}$ and $Eda^{Ta/Ta}$ embryos were obtained from $Eda^{Ta/+}$ females crossed with $Eda^{Ta/Y}$ males. E13.5 $Edar^{dlJ/dlJ}$ and $Edar^{dlJ/+}$ embryos were obtained from $Edar^{dlJ/+}$ females crossed with $Edar^{dlJ/dlJ}$ males. E15.5 and E17.5 $Eda^{Ta/Ta}$, $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$ embryos were obtained from homozygous females crossed with hemi-/homozygous males. In the text, $Eda^{Ta/Ta}$ refers to $Eda^{Ta/Ta}$ females and $Eda^{Ta/Y}$ males: $Edar^{dlJ/dlJ}$ and $Edaradd^{Cr/Cr}$ refer to homozygous animals. CD1 mice were bred to obtain stage matched control animals where required. Pregnant females were sacrificed by cervical dislocation and noon on the day of discovery of the vaginal plug was designated day 0.5 of development. Embryos were harvested and decapitated in phosphate buffered saline (PBS) for immediate fixation, or in complete culture medium (Advanced Dulbecco modified Eagle's minimal essential medium F-12 (DMEM-F12) (Gibco, Invitrogen) supplemented with 1% penicillin/streptomycin and 1% Glutamax (Gibco, Invitrogen)) for tongue culture experiments.

Genotyping

DNA was extracted by digestion of tail snip tissue in 0.5 mg/mL proteinase K at 55 °C overnight, followed by phenol chloroform purification and ethanol precipitation. Genotype of E13.5 WT, $Eda^{Ta/+}$ and $Eda^{Ta/Ta}$ embryos was identified by PCR analysis using primer sets specific for *Eda* (covering the exon 1 region deleted in $Eda^{Ta/Ta}$ and therefore failing to generate a product from Eda mutant DNA), Y chromosome and Actin (for positive control). Primer sequences were: EDA: 5′-AGGACAGTAGTCGCCTGT-3′ (forward), 5′-GCCGCCGCCCTTCCTAGG-3′ (reverse). Y chromosome: 5′-CTGGAGCTCTACAGTGATGA-3′ (forward), 5′-CAGTTACCAATCAACACATCAC-3′ (reverse). Actin: 5′-GCTTCTGAGATGTCTCTCTCT-3′ (forward), 5′-ACACAGGCTTTTGTAGGTTGC-3′ (reverse). These primers generated 509 bp (*Eda*), 343 bp (Y chromosome) and 159 bp (Actin) fragments.

The *Edar* mutant animals used in this study were of the $downless^{Jackson}$ strain which possess a G to A point mutation within the *Edar* death domain (Headon and Overbeek, 1999). Genotype of E13.5 $Edar^{dlJ/dlJ}$ and $Edar^{dlJ/+}$ embryos was determined by PCR analysis using *Edar* specific primers, the forward primer containing a one nucleotide mismatch from the template DNA. The mismatch enabled the generation of a TaqI restriction site unique to the WT sequence. Primer sequences were 5′-TGAAAACATGGCGCCACCTTGTC-3′ (forward, mismatch underlined), 5′-TCACTCACAGCTGTCGGTCGTG-3′ (reverse). These primers generated a 309 bp product. Overnight digestion of $Edar^{dlJ/dlJ}$ product with TaqI (Sigma) at 65 °C resulted in 142 bp and 167 bp fragments (due to a natural TaqI site within the PCR product), whereas restriction of $Edar^{dlJ/+}$ product resulted in fragments of 167 bp, 142 bp, 120 bp and 22 bp. Fragments were visualised on a 3% agarose gel. The mismatch primer was designed using dCAPS Finder 2.0 online software.

PCR cycling conditions were as follows: 94 °C 2 min 30 s, 94 °C 30 s, 54 °C 30 s, 72 °C 1 min for 36 cycles.

Tissue processing and sectioning

Tissues were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight and washed in PBS before dehydration in a methanol and isopropanol series, and clearing in 1,2,3,4-tetrahydronaphthalene. Adult heads were additionally subjected to decalcification in 1% PFA and 25% 0.5 M EDTA in PBS for 2 weeks prior to dehydration. Cleared samples were incubated in paraffin wax at 65 °C before embedding in moulds. Sections were cut to a thickness of 8 μm, mounted on glass slides (Superfrost Plus™, VWR International) and dried on a 42 °C hotblock overnight.

In situ hybridisation

Radioactive *in situ* hybridisation of ^{35}S-UTP-labelled *Edaradd* probe was performed as described in Tucker et al. (1999). *Edaradd* was linearized with Spe1 and transcribed with T3. Sections were counterstained with methyl green and photographed under darkfield. *In situ* hybridisation of dioxygenin-labelled *Shh*, *Ptc1*, *Erm* and *Nkx3.1* probes was performed according to modified Wilkinson procedures (Wilkinson, 1995) and sections were counterstained with eosin. *Shh* was linearized with EcoRI and transcribed with T7. *Nkx3.1* was linearized with EcoRV and transcribed with T7. *Ptc1* was linearized with BamHI and transcribed with T3. *Erm* was linearized with HindIII and transcribed with T7.

Histology staining

Sections were cleared with Histoclear™, rehydrated through an ethanol series and washed in dH_2O. Sections were stained with haemotoxylin and eosin (H&E) and differentiated in acid alcohol, or alternatively were stained with Sirius red in picric acid, Alcian blue and haemotoxylin (trichrome) where appropriate. After staining, sections were washed in dH_2O, dehydrated through an ethanol series, cleared in xylene and coverslipped with DPX mounting medium.

Organ culture

Tongues were dissected whole in complete culture medium and care was taken to remove all SMG tissue. Explants were mounted on membranes (BD Falcon cell culture inserts) and floated over culture medium in glass bottom dishes (MatTek Corporation). Medium for experimental explants was supplemented with recombinant Fc-EDA A1 (Gaide and Schneider, 2003) Fgf8b, or Shh-N peptide (R&D Systems). Explants were cultured in a 37 °C, 5% CO^2 incubator, photographed and the medium changed daily.

Analysis

Unpaired *t*-tests were performed for statistical comparison of groups of data and *p* values are shown in the figures. Data were analysed and converted to charts using Microsoft Office Excel 2007. The level of statistical significance was taken as $p<0.05$.

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Conflict of interest statement

The authors declare no conflicts of interest.

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