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Citation for published version:

Wells, K, Mou, C, Headon, D & Tucker, A 2010, 'Recombinant EDA or Sonic Hedgehog rescue the branching defect in Ectodysplasin A pathway mutant salivary glands in vitro.' *Developmental Dynamics*, vol. 239, no. 10, pp. 2674-84. DOI: 10.1002/dvdy.22406

Digital Object Identifier (DOI):

[10.1002/dvdy.22406](https://doi.org/10.1002/dvdy.22406)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Early version, also known as pre-print

Published In:

Developmental Dynamics

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Recombinant EDA or Sonic Hedgehog Rescue the Branching Defect in Ectodysplasin A Pathway Mutant Salivary Glands In Vitro

K.L. Wells,¹ C. Mou,² D.J. Headon,² and A.S. Tucker^{1*}

Hypohidrotic ectodermal dysplasia (HED) is characterized by defective ectodermal organ development. This includes the salivary glands (SGs), which have an important role in lubricating the oral cavity. In humans and mice, HED is caused by mutations in Ectodysplasin A (*Eda*) pathway genes. Various phenotypes of the mutant mouse *Eda^{Ta/Ta}*, which lacks the ligand *Eda*, can be rescued by maternal injection or in vitro culture supplementation with recombinant EDA. However, the response of the SGs to this treatment has not been investigated. Here, we show that the submandibular glands (SMGs) of *Eda^{Ta/Ta}* mice exhibit impaired branching morphogenesis, and that supplementation of *Eda^{Ta/Ta}* SMG explants with recombinant EDA rescues the defect. Supplementation of *Edar^{dlJ/dlJ}* SMGs with recombinant Sonic hedgehog (*Shh*) also rescues the defect, whereas treatment with recombinant *Fgf8* does not. This work is the first to test the ability of putative *Eda* target molecules to rescue *Eda* pathway mutant SMGs. *Developmental Dynamics* 239:2674–2684, 2010. © 2010 Wiley-Liss, Inc.

Key words: salivary gland; ectodermal dysplasia; *Eda*; branching morphogenesis; ectodermal organ development

Accepted 26 July 2010

INTRODUCTION

Mutations in Ectodysplasin A (*Eda*) pathway genes cause hypohidrotic ectodermal dysplasia (HED). Ectodermal dysplasia encompasses a group of disorders characterized by defective development of ectodermally derived structures such as the hair, teeth, and exocrine glands (Clarke et al., 1987). *EDA* encodes the tumor necrosis factor (TNF)-like ligand EDA A1 (Srivastava et al., 1997). EDA A1 signals by means of its receptor EDAR and an intracellular adaptor protein EDAR-ADD (Headon et al., 2001), and is thought to instigate downstream activation of NFκB (Kumar et al., 2001).

EDA can also undergo alternative splicing to produce EDA-A2 (Srivastava et al., 1997), although the biological function of this splice variant is unknown. 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 *Edar-add^{Cr/Cr}* (Srivastava et al., 1997; Monreal et al., 1999; Headon et al., 2001) exhibit a similar phenotype to

human HED in that the hair, teeth, and exocrine glands are impaired (Falconer et al., 1951; Grüneberg, 1965, 1971; Claxton, 1967; Blecher et al., 1983). The role of *Eda* signaling in hair and tooth development are thriving areas of research, whereas research into the role of *Eda* signaling in exocrine gland development, in particular that of the salivary glands (SGs), is relatively neglected and warrants further investigation.

SGs have an important role in providing lubrication for digestion and protection of the oral cavity. HED patients demonstrate reduced whole saliva flow (Nordgarden et al., 2001)

Additional Supporting Information may be found in the online version of this article.

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Grant sponsors: The UK Medical Research Council; National Foundation for Ectodermal Dysplasias.

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DOI 10.1002/dvdy.22406

Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

leading to xerostomia (dry mouth), further damage to an already compromised dentition and a reduced quality of life. Mammals have three pairs of major SGs that produce approximately 90% of total saliva (reviewed in Tucker, 2007): the submandibular (SMG) and sublingual (SLG) glands, situated under the tongue with ducts secreting into the floor of the mouth, and the parotid at the back of the mouth in between the upper and lower jaw, with ducts secreting saliva into the inner cheek.

Of the various SG types, the murine SMG is the most studied and its development has been depicted in stages (Jaskoll and Melnick, 1999). The developing SMG consists of ectodermally derived epithelial branches enclosed within a capsule of cranial neural crest derived mesenchyme (Jaskoll et al., 2002). The SMG is first visible at embryonic day (E) 11.5 (prebud stage) appearing as a thickening of the oral epithelium next to the tongue. By E12.5 (initial bud stage), the epithelial thickening has protruded into the underlying mesenchyme, forming a bud that proliferates and undergoes branching morphogenesis to form several branches by E14.5 (pseudoglandular stage). Lumina begin to form in the presumptive ducts and end buds (the presumptive acini) at approximately E15.5 (canalicular stage), and the first secretory product can be detected from around E17.5 (terminal differentiation stage; Melnick and Jaskoll, 2000).

Eda is expressed in the mesenchyme and *Edar* in the epithelium of the developing SMG at E13 and E14 (Pispa et al., 2003), although the expression of *Edaradd* has not been described. In *Eda^{Ta/Ta}* and *Eda^{dlJ/dlJ}* adults, the SMGs are hypoplastic with a reduced number of ductal and acinar structures (Blecher et al., 1983; Jaskoll et al., 2003). Regarding the developmental origin of this phenotype, the classical study of gland development in *Eda^{Ta/Ta}* states that the embryonic SMGs are unaffected (Grüneberg, 1971), but it has been reported recently that *Eda^{Ta/Ta}* SMGs exhibit reduced branching and developmental delay from E13 (Melnick et al., 2009). Here, we provide an analysis of *Eda^{Ta/Ta}* and wild-type (WT) littermate SMGs to build on this

data. We also study the SMGs of embryos heterozygous for *Eda* mutation, as female *Eda^{Ta/+}* carriers exhibit defective tooth development, particularly in the upper jaw (Charles et al., 2009).

Rescue of aspects of the *Eda^{Ta/Ta}* phenotype has been achieved using several methods. Transgenic overexpression of *Eda A1* driven by the CMV promoter leads to the rescue of the *Eda^{Ta/Ta}* hair and sweat gland phenotypes as well as hypodontia (Srivastava et al., 2001). Similarly, expression of an *Eda A1* transgene in a tetracycline regulated conditional mouse model rescued sweat glands and hair development in *Eda^{Ta/Ta}*, but the developmental timing of transgene activation is crucial and specific for each organ (Cui et al., 2009). Injection of a recombinant form of *EDA A1* given to pregnant *Eda^{Ta/Ta}* mice in a series beginning at E11 leads to the almost complete rescue of the organs affected in the developing embryos (Gaide and Schneider, 2003), although the effect on the SGs was not documented. In vitro, supplementation of *Eda^{Ta/Ta}* skin cultures with recombinant *EDA A1* rescues hair placode development (Mustonen et al., 2004), while *EDA A1* addition to WT SMG cultures increases branching and enhances NF κ B activation (Jaskoll et al., 2003). It follows that it may be possible to rescue *Eda^{Ta/Ta}* SMGs in culture by recombinant *EDA A1* supplementation, and here we show that this is achievable during both early and later branching morphogenesis, an encouraging finding for future attempts to correct SG defects in HED patients.

Recent years have seen considerable progress in identifying the genes regulated by *Eda* signaling. Notably, treatment of embryonic *Eda^{Ta/Ta}* skin cultures with recombinant *EDA A1* results in rapid and significant upregulation of *Shh* (Pummila et al., 2007). Furthermore, recent quantitative polymerase chain reaction (qPCR) profiling has revealed downregulation of *Shh* in *Eda^{Ta/Ta}* SMGs in comparison with WT SMGs (Melnick et al., 2009). We have sought to build on these data by testing the ability of exogenous Sonic hedgehog (*Shh*) to rescue *Eda* pathway mutant SMGs in vitro. We find that

supplementation of *Edar^{dlJ/dlJ}* SMGs with recombinant *Shh* rescues the observed defects, whereas treatment with recombinant *Fgf8*, another putative *Eda* target found to be significantly down-regulated in *Eda^{Ta/Ta}* SMGs (Melnick et al., 2009), does not rescue the glands.

RESULTS

Edar and *Edaradd* Are Expressed From the Earliest Stages of SMG Development

The expression of *Eda* and *Edar* has been investigated at E13 and E14 in the SMGs (Pispa et al., 2003), but expression during the earliest stages of SMG development has not been studied, nor has *Edaradd* expression been assessed at any stage. Therefore, we investigated the expression of *Eda* pathway genes at early stages of SMG development by in situ hybridization, concentrating on the expression of *Edar* and *Edaradd*, because the expression of these genes likely reflects the location at which the pathway is acting. At E11.5, *Edar* was found to be strongly expressed in the oral epithelial thickenings of the developing SMG and in the developing SMG bud at E12.5 (Fig. 1A,B). *Edaradd* expression was also confined to the epithelium of the developing SMG from E12.5–E15.5 (Fig. 1C–F).

Eda^{Ta/Ta} and *Eda^{Ta/+}* SMGs Exhibit Abnormal Branching Morphogenesis In Vitro

Eda^{Ta/Ta} adult SMGs are hypoplastic with reduced ducts and acini (Blecher et al., 1983; Jaskoll et al., 2003). To determine the embryonic stage at which this defect originates, we chose to assess the mutant glands after dissection rather than in histological sections to evaluate the size and morphology of the glands accurately. E13.5 WT, *Eda^{Ta/+}*, and *Eda^{Ta/Ta}* littermate SMGs (n = 35 pairs in total from 3 litters) were examined, and we found no significant difference in branch number between any of the genotypes at this stage. However, we found a high level of morphological variation among the *Eda^{Ta/Ta}* SMGs, and were able to define 3 morphotypes based on developmental stage and

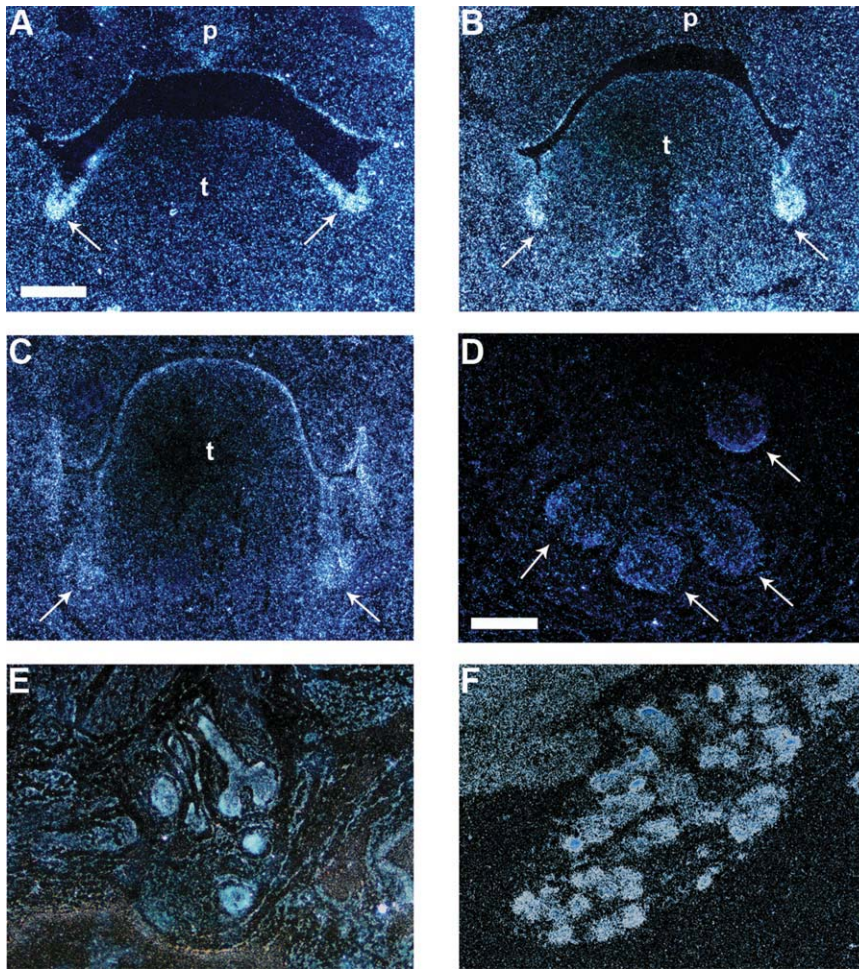


Fig. 1.

Fig. 1. *Edar* and *Edaradd* are expressed from the earliest stages of submandibular gland (SMG) development. ³⁵S in situ hybridization of *Edar* (A,B) and *Edaradd* (C–G) on frontal sections. **A:** Embryonic day (E) 11.5 oral cavity, showing *Edar* expression in the presumptive SMG epithelial thickenings (arrows). **B:** E12.5 oral cavity. *Edar* is expressed in the SMG epithelial buds (arrows). **C:** E12.5 oral cavity, showing *Edaradd* expression in the SMG epithelial buds (arrows). **D–F:** *Edaradd* expression in the SMG epithelial branches at E13.5 (D), E14.5 (E), and E15.5 (F). t, tongue; p, roof of nasal passage. Scale bar = 200 μ m in A (applies in A–C,E,F); 100 μ m in D.

number of branches (Fig. 2A–C). Although WT SMGs demonstrated mild variability, and of a level normally expected in litters of WT embryos, we observed a much wider dispersion of *Eda*^{Ta/Ta} SMGs with respect to their branch number (Fig. 2D). WT and *Eda*^{Ta/Ta} SMGs within the same litter regularly fell into the same morphotype group and were indistinguishable from each other in terms of size and branch number (compare Figs. 3A and 3B, first panels from left). This indicates that *Eda*^{Ta/Ta} and *Eda*^{Ta/+} SMGs exhibit high morphological variation in comparison with their WT littermates and that, at this stage of development, it is unfeasible to attempt to identify an

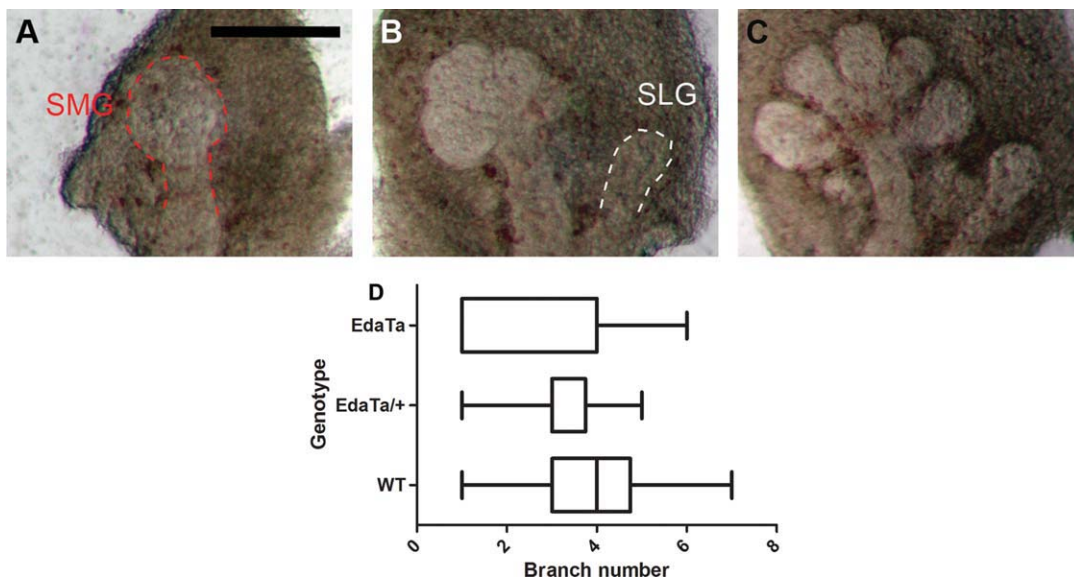


Fig. 2. Embryonic day (E) 13.5 *Eda*^{Ta/Ta} submandibular glands (SMGs) and sublingual glands (SLGs) exhibit high morphological variation. A–C: E13.5 *Eda*^{Ta/Ta} SMG/SLGs from the same litter representative of three morphotypes defined by developmental stage and branch number. **A:** E13.5 bud morphotype. SMG outlined in red. **B:** E13.5 intermediate morphotype indicating cleft formation, which is absent in A. SLG is outlined in white. **C:** E13.5 Pseudoglandular morphotype with development of distinct SMG branches in comparison to (B). **D:** Box and whisker plot showing dispersion of branch number data for E13.5 SMGs from the three genotype groups. The interquartile range (indicated by box) of the *Eda*^{Ta/Ta} group of SMGs is wider than that of the WT group, indicating greater variability in E13.5 *Eda*^{Ta/Ta} SMG branch number compared with WTs. Scale bar in A (for A–C) = 200 μ m.

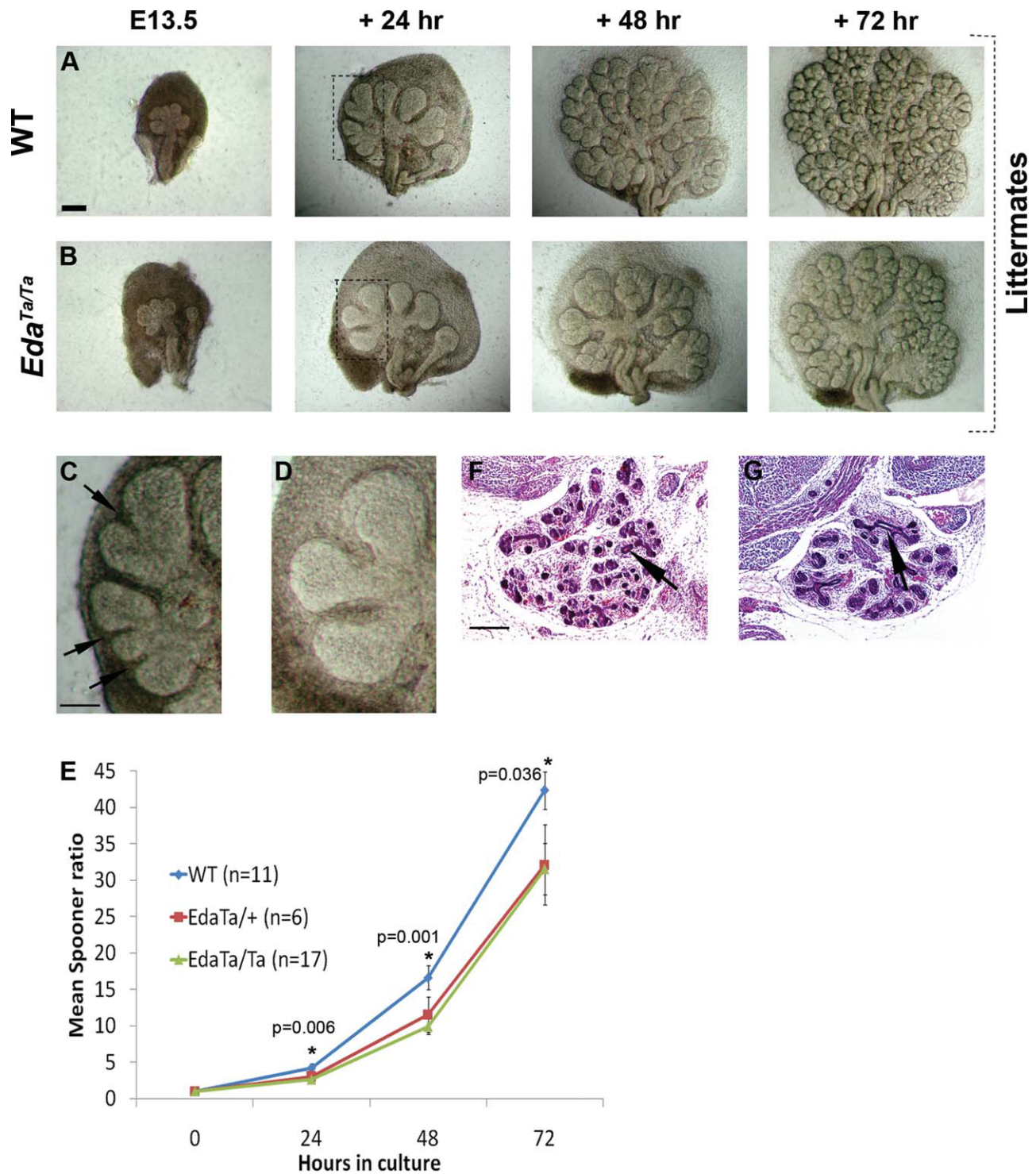


Fig. 3. Embryonic day (E) 13.5 *Eda^{Ta/Ta}* submandibular glands (SMGs) exhibit defective branching morphogenesis. **A,B:** Stage matched E13.5 wild-type (WT; A) and *Eda^{Ta/Ta}* (B) SMGs from littermate embryos cultured for 72 hr. After 24 hr, *Eda^{Ta/Ta}* SMGs exhibit visibly less branching when compared with WT, and a similar trend is evident at 48 and 72 hr. **C:** High magnification of dashed box in (A +24 hr) showing cleft formation in WT SMG. **D:** High magnification of dashed box in (B +24 hr) showing lack of cleft formation in *Eda^{Ta/Ta}* SMG in comparison to (C). **E:** Chart showing mean Spooner ratios of WT, *Eda^{Ta/+}*, and *Eda^{Ta/Ta}* groups at each time point in culture. The Spooner ratio is a measure of branching over time, calculated by dividing number of buds present on day \times by number of buds in the same explant at day 0. Error bars depict one standard error \pm mean. An asterisk denotes statistical significance (unpaired *t*-test) between *Eda^{Ta/Ta}* and WT means. **F:** Hematoxylin and eosin-stained wax section of E15.5 WT SMG. **G:** Representative *Eda^{Ta/Ta}* SMG (n = 6) stage matched to F. Lumen formation (arrows) is unaffected in the mutant. Scale bar = 200 μ m in A (applies in A,B), 100 μ m in C (applies in C,D) in F (applies in F,G).

Eda^{Ta/Ta} SMG phenotype. Furthermore, *Eda* appears to be consistently dispensable for initiation and in many cases for the early development of the SMG.

To determine whether *Eda* signaling plays a role in SMG branching morphogenesis, we next compared the development of precisely stage matched E13.5 WT and *Eda^{Ta/Ta}* littermate SMGs for 72 hr in vitro. We found that *Eda^{Ta/Ta}* SMGs exhibited visibly less branching when compared with WTs. The effect is apparent after 24 hr of culture when the *Eda^{Ta/Ta}* gland displays less cleft formation than WTs (Fig. 3A–D). After 72 hr of development, *Eda^{Ta/Ta}* SMGs had formed markedly fewer branches in comparison with stage matched WT littermates (Fig. 3A,B). This observation is supported by histological analysis of E15 WT and *Eda^{Ta/Ta}* SMGs (Fig. 3F,G). In the WT SMG at E15, the epithelial branches begin to hollow out to form lumen (Fig. 3F); ductal lumen were found to be developing as normal in the mutant SMGs at E15 (Fig. 3G), indicating that the branching defect is not due to overall developmental delay of the gland.

Traditionally, quantification of SMG branching is achieved by counting the number of buds present in SMG explants at a defined time point, and dividing this by the number of buds present at the onset of the culture period. The result is termed the Spooner ratio (Spooner and Bassett, 1989) and gives a measure of the extent of branching that has taken place in a particular explant over a defined time period. To quantify the visible differences between WT and *Eda^{Ta/Ta}* SMGs in vitro, mean Spooner ratios for the WT, *Eda^{Ta/+}* and *Eda^{Ta/Ta}* groups were compared at each time point (Fig. 3E). After 24 hr in culture, *Eda^{Ta/Ta}* SMGs exhibited a 38% lower Spooner ratio than WTs. Subsequently the branching defect was not recovered, with *Eda^{Ta/Ta}* SMGs exhibiting a significant 41% lower Spooner ratio than WTs at 48 hr. This indicates that the phenotype observed in *Eda^{Ta/Ta}* SMGs stems from an early defect in branching morphogenesis. Of interest, the *Eda^{Ta/+}* SMGs followed a similar developmental trend to the *Eda^{Ta/Ta}* SMGs; mean Spooner ratios for the *Eda^{Ta/+}* group were not significantly

different from the *Eda^{Ta/Ta}* SMG group at any time point (Fig. 3E).

Recombinant EDA A1 Rescues Branching in *Eda^{Ta/Ta}* SMGs In Vitro

Recombinant EDA A1 supplementation rescues primary hair follicle development in *Eda^{Ta/Ta}* skin explants (Mustonen et al., 2004). We attempted to rescue the branching defect identified in embryonic *Eda^{Ta/Ta}* and *Eda^{Ta/+}* SMGs by EDA A1 supplementation in vitro. E13.5 SMGs were initially selected because this stage precedes the appearance of the branching defect. We chose to supplement the glands with 100 ng/ml EDA A1 because this concentration rescues primary hair placode development in *Eda^{Ta/Ta}* skin in vitro (Mustonen et al., 2004).

Before attempting SMG rescue experiments, it was necessary to test our batch of EDA A1 to confirm functionality. Dorsal skin explants from E13.5 *Eda^{Ta/Ta}* embryos were treated with EDA A1 for 24 hr to rescue primary hair placodes, which do not develop in *Eda^{Ta/Ta}* (Mustonen et al., 2004; Mou et al., 2006). While primary hair placodes, as detected by *Shh* expression, remained absent in untreated *Eda^{Ta/Ta}* skin, rescued placodes were detected in EDA A1-treated *Eda^{Ta/Ta}* skins (Supp. Fig. S1A–C, which is available online). For the SMG rescue experiments, *Eda^{Ta/Ta}* SMGs from the same embryo were reserved in pairs. From each pair, one SMG was treated with 100 ng/ml EDA A1 and its counterpart from the same embryo was cultured in the absence of EDA A1. After 72 hr, E13.5 SMGs supplemented with 100 ng/ml EDA A1 demonstrated visibly augmented branching (Fig. 4B) compared with their unsupplemented control pairs (Fig. 4A) and appeared comparable in branch number to WT littermate SMGs (Fig. 4B,C). In fact, more cleft formation and budding was visible in the treated glands (Fig. 4E) compared with their control pairs (Fig. 4D) as early as 24 hr into the culture period. Calculation of Spooner ratio and paired *t*-test revealed a statistically significant 36% ($P = 0.02$) mean increase in branching of the supplemented explants compared with their un-

plemented pairs after 24 hr, and an 84% ($P = 0.001$) mean increase after 48 hr. Comparison of Spooner ratios at all time points between the WT, treated, and untreated groups of glands showed that the branching defect seen in *Eda^{Ta/Ta}* SMGs was fully rescued after only 24 hr of supplementation with 100 ng/ml EDA A1 (Fig. 4F). With addition of fresh EDA A1 daily, the rescue was maintained with an absence of excess branching or negative effects for a further 48 hr at which point the experiment was terminated. Rescue of the branching phenotype and an absence of negative effects was apparent in the treated SMGs from all morphotype groups, indicating that *Eda^{Ta/Ta}* SMGs can be treated with 100 ng/ml EDA A1 from an early stage of development to achieve rescue.

Recombinant EDA A1-mediated rescue of other ectodermal structures affected by the *Eda* mutation is highly stage dependent. To determine whether the SMGs could be rescued later in development, E15.5 *Eda^{Ta/Ta}* SMGs were cultured with 500 ng/ml EDA A1 (a high concentration to ensure adequate penetration of the larger explants). After 24 hr, the EDA A1-treated explants exhibited a mean 19% greater ($P = 0.01$, paired *t*-test) Spooner ratio compared with their untreated pairs (Fig. 5A–D). *Eda^{Ta/Ta}* SMGs, therefore, remain responsive to exogenous recombinant EDA A1 subsequent to the manifestation of the branching defect, suggesting that the possibility of recombinant EDA A1-mediated rescue is not restricted to early SMG branching morphogenesis.

Recombinant Shh-N Rescues Branching in *Edar^{dlJ/dlJ}* SMGs In Vitro

qPCR profiling has revealed down-regulation of *Shh* in *Eda^{Ta/Ta}* SMGs in comparison with WT SMGs (Melnick et al., 2009), and treatment of embryonic *Eda^{Ta/Ta}* skin cultures with recombinant EDA A1 results in up-regulation of *Shh* (Pummila et al., 2007). We hypothesized that application of recombinant Shh to *Eda* pathway mutant SMGs explants would rescue the branching defect. Both *Eda^{Ta/Ta}* and *Edar^{dlJ/dlJ}* adult SMGs are characterized by hypoplasia and a

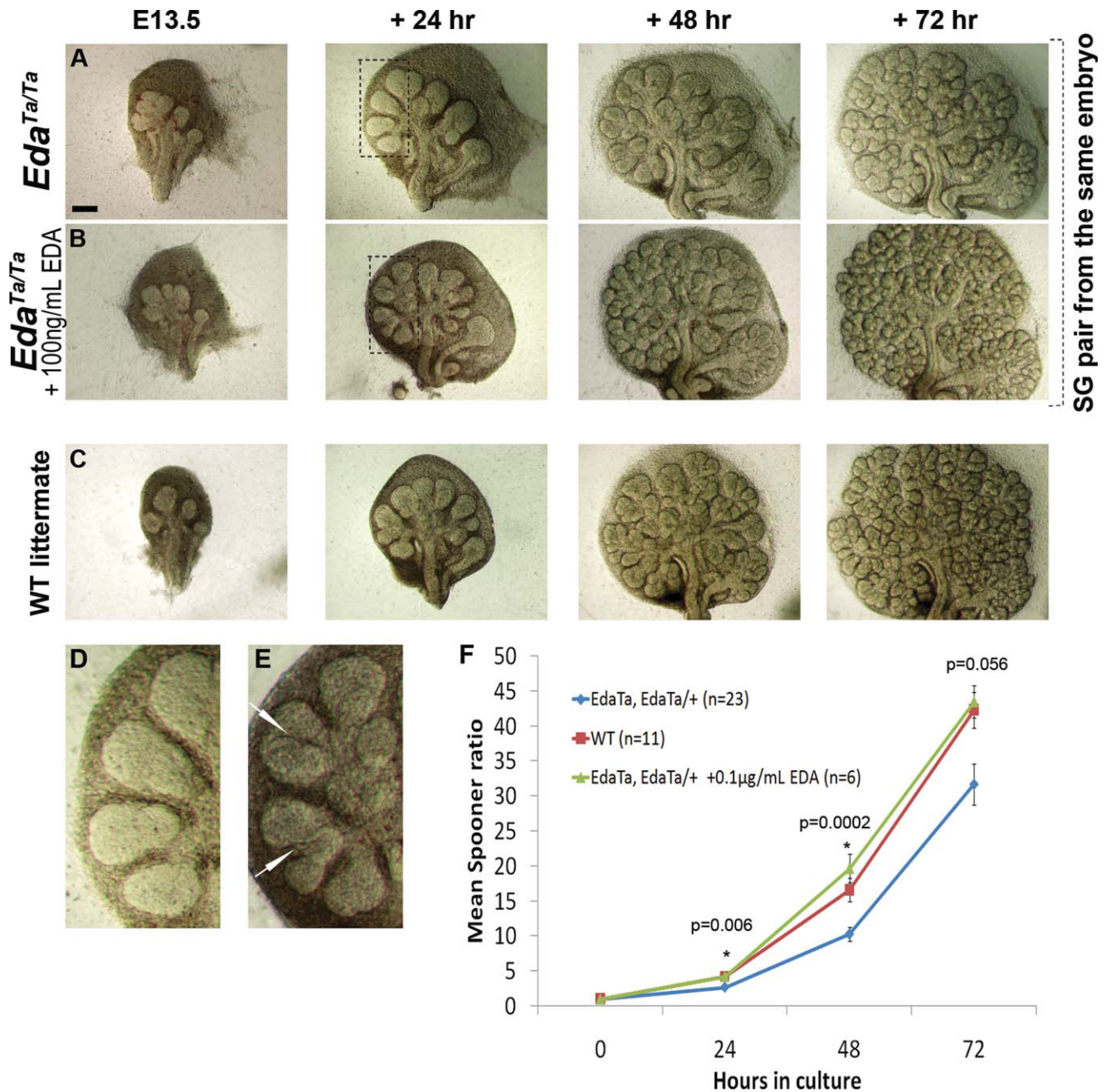


Fig. 4. 100 ng/ml exogenous recombinant EDA A1 rescues branching in embryonic day (E) 13.5 *Eda^{Ta/Ta}* submandibular glands (SMGs) in vitro. E13.5 *Eda^{Ta/Ta}* SMG pair (A,B) and E13.5 wild-type (WT) littermate SMG (C) cultured for 72 hr. **A,B:** Untreated *Eda^{Ta/Ta}* SMG (A), and *Eda^{Ta/Ta}* SMG (B) from the same embryo as A treated with 100 ng/ml EDA A1. Branching is increased in the treated SMG from 24 hr in comparison with A. **C:** WT SMG, littermate of A and B. Branching is comparable to B. **D:** High magnification of dashed box in (A +24 hr). **E:** High magnification of dashed box in (B +24 hr) showing increased budding and cleft formation in treated *Eda^{Ta/Ta}* SMG in comparison with D. **F:** Chart showing mean Spooner ratios of WT, untreated and 100 ng/ml EDA A1-treated groups at each time point in culture. An asterisk denotes statistical significance between untreated *Eda^{Ta/Ta}* / *Eda^{Ta/+}* group and 100 ng/ml-treated group means (unpaired *t*-test). Error bars depict one standard error \pm mean. Scale bar = 200 μ m in A (applies in A–C), 100 μ m in D (applies in D,E).

reduction in acinar and ductal structures (Jaskoll et al., 2003; data not shown). For our downstream rescue experiments, we moved to *Edar^{d1J/d1J}* SMGs. These mice lack functional *Edar*, and we considered them the best system for these experiments

because the possibility of activating signaling downstream of the *Eda* pathway through *Edar* is abolished.

Before attempting rescue with Shh-N, it was necessary to confirm activation of the expected developmental pathway by Shh-N in our SMG cul-

tures. E13.5 WT SMG explants were cultured for 24 hr with 1.5 μ g/ml Shh-N, a concentration determined by dose response experiment to preclude toxicity on the developing gland (data not shown) and processed for whole-mount in situ hybridization of *Gli1* or

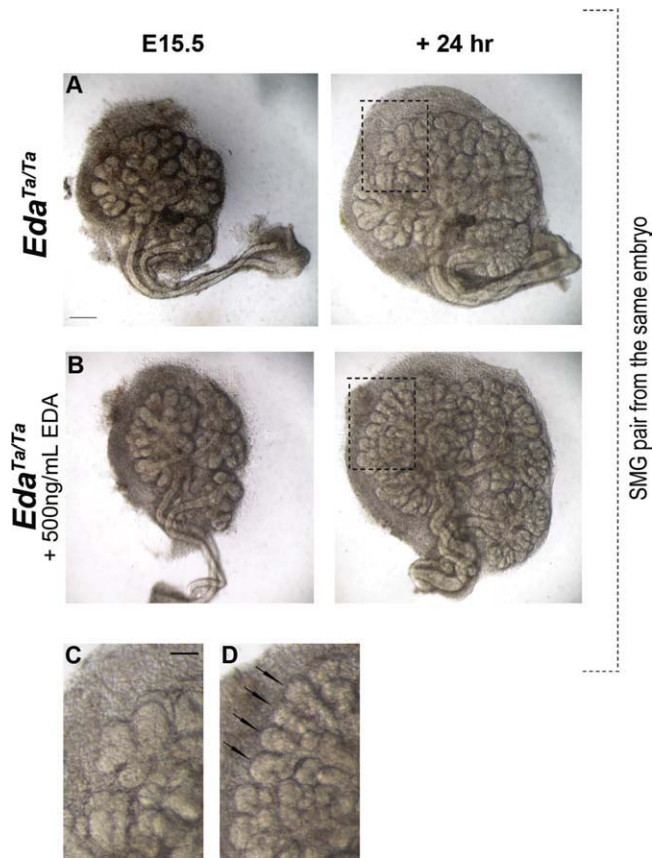


Fig. 5. Exogenous EDA A1 rescues branching in embryonic day (E) 15.5 *Eda^{Ta/Ta}* submandibular glands (SMGs) in vitro. E15.5 *Eda^{Ta/Ta}* SMG pair (n = 6). **A:** Untreated *Eda^{Ta/Ta}* SMG. **B:** SMG pair to A treated with 500 ng/ml EDA A1, showing more branch formation in comparison to A after 24 hrs. **C:** High magnification of dashed box in (A +24 hr). **D:** High magnification of dashed box in (B +24 hr), indicating more branch formation (arrows) in developing *Eda^{Ta/Ta}* SMG with EDA A1 treatment in comparison to C. Scale bar = 200 μ m in A (applies in A,B), 100 μ m in C (applies in C,D).

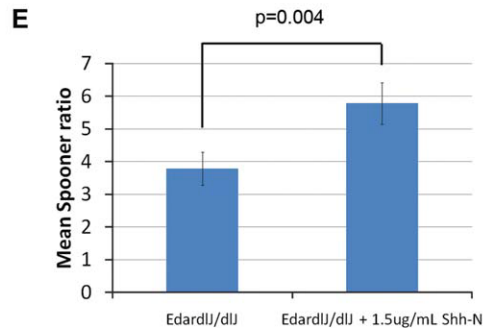
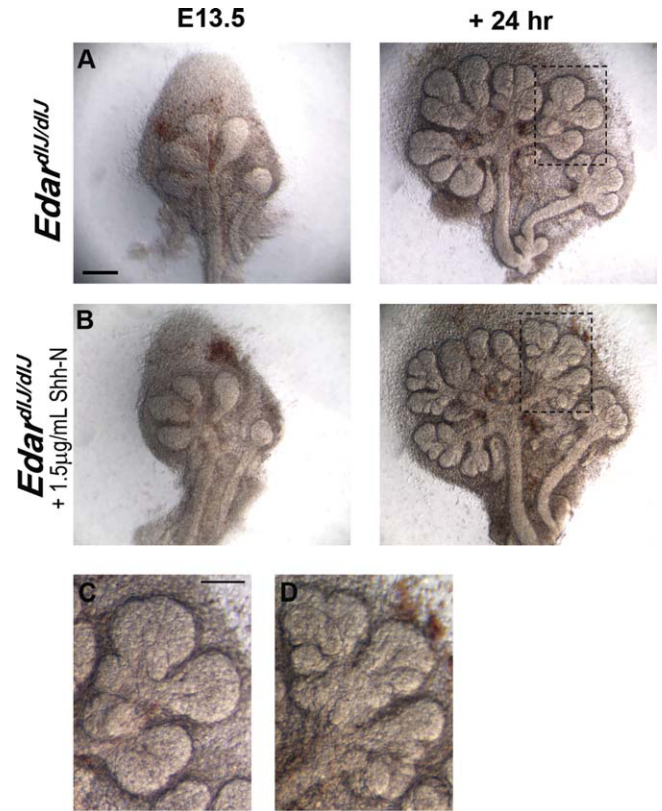


Fig. 6.

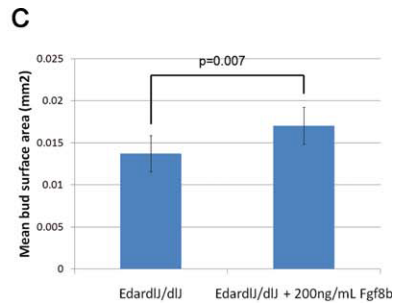
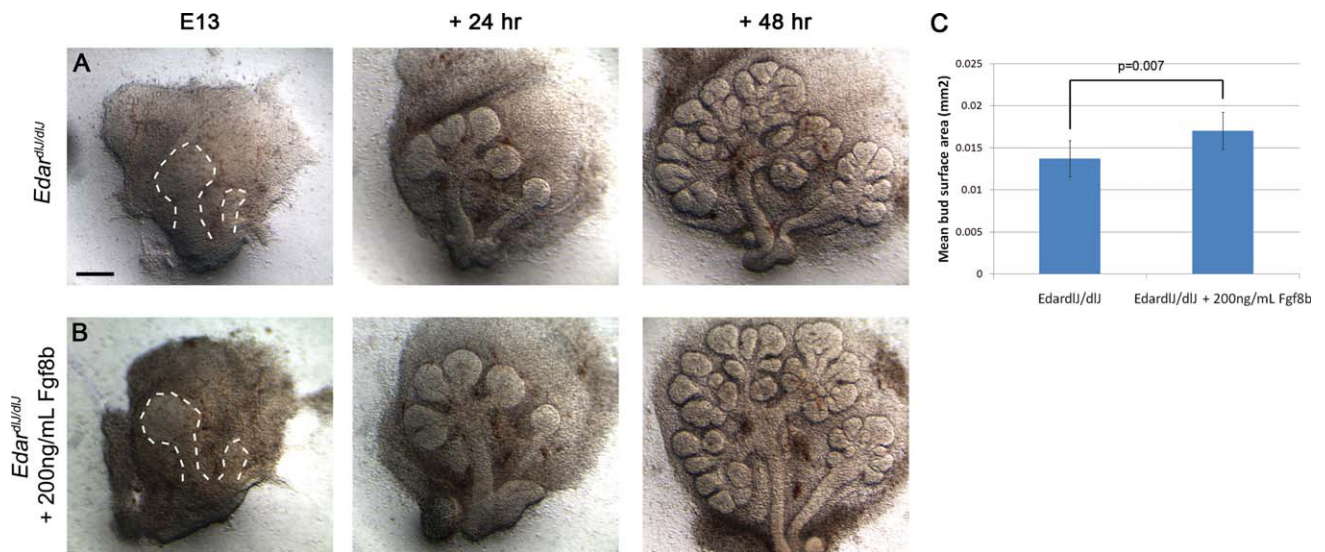


Fig. 7.

Ptc1, known transcriptional targets of Shh signaling (Ingham and McMahon, 2001). Up-regulation of both genes was observed in explants treated with Shh-N (Supp. Fig. S2A–D). Within the same period of color development, control SMGs showed no signal (Shh pathway components are normally expressed at very low levels in the SMGs). For the rescue experiments, E13.5 *Edar^{dlJ/dlJ}* SMGs were cultured for 24 hr with 1.5 μ g/ml Shh-N peptide. After 24 hr in culture, the Shh-N-treated explants exhibited a mean 58% greater ($P = 0.004$, paired *t*-test) Spooner ratio compared with their untreated pairs (Fig. 6), an improved increase in branching on that achieved by application of EDA A1 to E13.5 *Eda^{Ta/Ta}* SMGs for 24 hr (36%). Exogenous Shh is, therefore, sufficient to rescue the branching phenotype in *Edar^{dlJ/dlJ}* SMG explants, and like EDA A1-mediated rescue, is successful within 24 hr.

Fgf8 is a potential target of Eda signaling in the SMGs, because qPCR has revealed down-regulation of *Fgf8* in *Eda^{Ta/Ta}* SMGs (Melnick et al., 2009). We tested whether application of exogenous *Fgf8* was sufficient to rescue *Edar^{dlJ/dlJ}* SMGs in a similar manner to EDA A1 and Shh. E13.5 *Edar^{dlJ/dlJ}* SMGs were treated with 200 ng/ml *Fgf8*, a concentration shown by Jaskoll et al. (2004b) to increase branching in WT SMG explants after 72 hr in culture. After 24 hr in culture, we observed no difference in branch number between our *Fgf8*-treated *Edar^{dlJ/dlJ}* SMGs and their untreated pairs (Fig. 7). We allowed some of the explants to continue to develop for a further 48 hr and found no change in branch num-

ber in response to *Fgf8* treatment, although we noticed that the treated explants appeared larger overall than their untreated pairs (Fig. 7A,B). We quantified this by measuring the surface area of the epithelial portion of the explant, and dividing this value by branch number, to give a mean surface area per end bud. We found that the buds in the *Fgf8*-treated explants were a mean 24% larger than those of their untreated pair explants ($P = 0.007$, paired *t*-test; Fig. 7C). *Fgf8* application alone is, therefore, not sufficient to rescue branching in *Edar^{dlJ/dlJ}* SMGs, but appears to increase end bud size.

DISCUSSION

Eda Signaling Is Required for Normal SMG Branching Morphogenesis

Analysis of the expression of *Edar* and *Edaradd* revealed that these key constituents of the Eda pathway are expressed from the earliest stages of SMG development—earlier than previously reported. *Edar* is expressed in the thickening and budding oral epithelium of the developing SMG, which in terms of the location of expression agrees with the work of Pispas et al. (2003) who demonstrated *Edar* expression in the epithelium of the branching SMG at E13 and E14. In the present work, the expression of *Edaradd* is investigated for the first time in the developing SMG, and is shown to be confined to the epithelium from early stages of development through branching morphogenesis. The expression of *Edaradd* mirrors that of *Edar*, suggesting that *Edaradd* interacts with *Edar* in the developing SMGs as in other ectodermal

organs such as the tooth and hair follicles (Headon et al., 2001).

Given that the Eda pathway is expressed from the earliest stages of SMG development, it was reasonable to expect the appearance of a defect early in the development of the SMG. We show that E13.5 *Eda^{Ta/Ta}* SMGs in fact exhibit high morphological variation in comparison with their WT littermates. This variability is not surprising given that a similar phenomenon exists with respect to the *Eda^{Ta/Ta}* tooth phenotype; the cusp morphology of the teeth is variable, and the third molar is missing in some but not all cases (Grüneberg, 1967). The palatal rugae of *Eda^{Ta/Ta}* mice have also been shown to exhibit high phenotypic variability in comparison with WT (Charles et al., 2007). A similar high variability in the *Eda^{Ta/Ta}* SMG may explain why the original studies by Grüneberg (1971) found that the embryonic major SGs were unaffected, whereas results from Melnick et al. (2009) show that the *Eda^{Ta/Ta}* SMG is characterized by hypoplasia and developmental delay from E13.

Our analyses of E13.5 *Eda^{Ta/Ta}* SMGs and their WT littermates in culture show that the pathway is not required for initiation of the SMGs; rather the overarched defect is a failure of normal branching morphogenesis, confirmed by our observations of the development of stage matched *Eda^{Ta/Ta}* and WT littermate SMGs in vitro. The concept that the SMGs do not require Eda signaling at an early stage of development fits with data from other ectodermal organs; the tooth and secondary/tertiary hair follicles, despite their abnormal development in the absence of Eda signaling, do not require Eda signaling for

Fig. 6. Exogenous Sonic hedgehog (Shh) rescues branching in *Edar^{dlJ/dlJ}* submandibular glands (SMGs) in vitro. Embryonic day (E) 13.5 *Edar^{dlJ/dlJ}* SMG pair ($n = 5$). **A:** Control *Edar^{dlJ/dlJ}* SMG. **B:** SMG pair to A treated with 1.5 μ g/ml Shh-N, showing more branch formation in comparison to (A) after 24 hrs. **C:** High magnification of dashed box in (A +24 hr). **D:** High magnification of dashed box in (B +24 hr), indicating more cleft and branch formation in comparison to C. **E:** Chart showing mean Spooner ratios of untreated and 1.5 μ g/ml Shh-N-treated groups after 24 hr in culture. Significance measured by paired *t*-test. Error bars depict one standard error \pm mean. Scale bar = 200 μ m in A (applies in A,B), 100 μ m in C (applies in C,D).

Fig. 7. Exogenous *Fgf8* does not rescue branching in *Edar^{dlJ/dlJ}* submandibular glands (SMGs) in vitro. Embryonic day (E) 13.5 *Edar^{dlJ/dlJ}* SMG pair ($n = 5$). **A:** Control *Edar^{dlJ/dlJ}* SMG. **B:** SMG pair to A treated with 200 ng/ml *Fgf8*, indicating enlargement of explant at 48 hr in comparison with A. SMG and SLG epithelium outlined by dotted line at E13. **C:** Chart showing mean single bud size of control and *Fgf8*-treated explants after 48 hr in culture. Significance measured by paired *t*-test. Error bars depict one standard error \pm mean. Scale bar = 200 μ m in A (applies in A,B).

initiation (Pispa et al., 1999; Tucker et al., 2000; Laurikkala et al., 2002). These data, along with the present work, suggest some redundancy of the pathway. The TNF receptor Troy acts redundantly with Edar in secondary hair follicle development (Pispa et al., 2008) and it would be interesting to investigate the role of Troy in SMG development. We also show that embryonic *Eda^{Ta/Ta}* and *Eda^{Ta/+}* SMGs exhibit a similar branching defect. It can be hypothesized that the X-chromosome inactivation effect in female heterozygous carriers of the X-linked *Eda* mutation leads to a reduced level of Eda availability in *Eda^{Ta/+}* SMGs in comparison with WTs. This difference in expression level appears to have great consequences in SMG development, and this idea fits with data showing that human female carriers of XL-HED exhibit reduced saliva flow (Lexner et al., 2007).

***Eda^{Ta/Ta}* SMGs Can Be Rescued With Recombinant EDA A1 In Vitro**

Supplementation of E13.5 *Eda^{Ta/Ta}* SMGs with 100 ng/ml EDA A1 results in rescue of the branching phenotype after 24 hr, with EDA A1 supplementation bringing about increased epithelial clefting and branch elongation. This agrees with data showing that exogenous EDA A1 applied to E14 WT SMGs in culture increases branching (Jaskoll et al., 2003). We also show that recombinant EDA A1-mediated rescue is achievable later in branching morphogenesis at E15.5. Recombinant EDA A1-mediated rescue of other ectodermal structures affected by *Eda* mutation is highly stage dependent. For example, in experiments whereby the *Eda^{Ta/Ta}* phenotype was rescued by maternal injection of recombinant EDA A1, molar morphogenesis could only be fully rescued by injections administered from early in development (Gaide and Schneider, 2003). Our results show that the developing SMGs, unlike the developing teeth, are still able to respond to EDA A1 supplementation after the appearance of the *Eda^{Ta/Ta}* phenotype, an encouraging finding for future attempts to correct SG defects in HED patients.

***Edar^{dlJ/dlJ}* SMGs Can Be Rescued With Exogenous Shh In Vitro**

Shh signaling is essential for SMG development; the SMGs of *Shh^{-/-}* embryos do not develop beyond the onset of branching morphogenesis, and cyclopamine treatment of WT SMG explants results in less branching (Jaskoll et al., 2004a). Here, we show that exogenous Shh is sufficient to rescue the branching phenotype in *Edar^{dlJ/dlJ}* and can do so in the same time scale as EDA A1. We cannot discount the possibility that Shh-mediated rescue of *Edar^{dlJ/dlJ}* SMGs takes place by means of a mechanism independent of signaling downstream of Eda. However, taken together with previous studies showing that treatment of embryonic *Eda^{Ta/Ta}* skin cultures with recombinant EDA A1 results in up-regulation of *Shh* (Pummila et al., 2007) and that *Shh* is down-regulated in *Eda^{Ta/Ta}* SMGs (Melnick et al., 2009), our results provide strong additional evidence that *Shh* is an important downstream target of Eda signaling in the SMGs. *Eda^{Ta/Ta}* mice lack primary hair follicles, yet treatment of *Eda^{Ta/Ta}* skin cultures with exogenous Shh does not rescue their initiation (Pummila et al., 2007), consistent with a later role for Shh in skin appendage development. In some cases, however, *Shh* overexpression is associated with tumor formation in the skin (Oro et al., 1997). In our experiments, the morphology of SMGs treated with Shh appeared normal, with no indication of ectopic SMG formation or tumorigenesis. Therefore, our results are also consistent with a role for Shh in later stage development of ectodermal appendages.

qPCR data have indicated that *Fgf8* is a target of Eda signaling in the SMGs (Melnick et al., 2009). We were unable to demonstrate rescue of *Edar^{dlJ/dlJ}* SMG explants by *Fgf8* supplementation, although we observed an increase in gland size. 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 normally independent of Eda signaling, such as an increase in epithelial proliferation. It is possible that the effects we observed on

Fgf8 supplementation of *Edar^{dlJ/dlJ}* SMGs are due to a similarly generalized increase in proliferation, but it also seems plausible that *Fgf8* is unable to act alone in the formation of branches and requires other factors for normal morphogenesis. It seems likely that Eda signaling controls a variety of different transcriptional targets in the SMGs, and future work which identifies how these different factors act in concert to control branching morphogenesis will be of interest.

EXPERIMENTAL PROCEDURES

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 *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 embryos where required. Pregnant females were killed by cervical dislocation and noon on the day of discovery of the vaginal plug was designated day 0.5 of development. Embryos were harvested, staged according to Theiler (1989) 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 organ 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}* that failed to generate a product from *Eda* mutant DNA), Y chromosome, and Actin (for positive control). Primer sequences were as follows: *EDA*: 5'-AGGACAGTAGTC-GCCTGT-3' (forward), 5'-GCCGCCG-CCCTTCCTAGG-3' (reverse); Y chromosome: 5'-CTGGAGCTCTACAGTG-ATGA-3' (forward), 5'-CAGTTACCA-ATCAACACATCAC-3' (reverse); Actin: 5'-GCTTCTGAGATGCTCTCTCT-3' (forward), 5'-ACACAGGCTTTTGTA-GGTTGC-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 recessive 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'-TCACTCACAGCTGTCGGTCTCGTG-3' (reverse). These primers generated a 309-bp product. Overnight restriction 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 visualized on a 3% agarose gel. The mismatch primer was designed using dCAPS Finder 2.0 online software.

PCR cycling conditions were: 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 at 4°C overnight and washed in PBS before dehydration in a methanol and isopropanol series, and clearing in 1,2,3,4-tetrahydronaphthalene. 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 Hybridization

Radioactive in situ hybridization of ³⁵S-UTP-labeled *Edar* and *Edaradd* probes was performed as described in Tucker et al. (1999). *Edar* was linearized with *Bss* HI and transcribed with T7. *Edaradd* was linearized with *Spe* I and transcribed with T3. Sections were counterstained with methyl green and photographed under dark-field.

Whole-mount in situ hybridization of digoxigenin-labeled *Ptc1* and *Gli1* probes was performed according to Wilkinson (1995). For reliable comparison of expression levels between treated and control glands, explants were subjected to simultaneous and identical processing and color development. Color was developed for 5 hr (when a strong signal became apparent in *Shh*-N-treated explants) before photographing. Three explants were processed per treatment group and probe, and all explants within each group showed similar results. *Gli1* was linearized with *Not*I and transcribed with T3. *Ptc1* was linearized with *Bam* HI and transcribed with T3.

Whole-mount in situ hybridization of a digoxigenin-labeled *Shh* probe on skin explants was performed as described in Mou et al. (2006).

Histology Staining

Sections were cleared with HistoClear, rehydrated through an ethanol series and washed in dH₂O. Sections were stained with hematoxylin and eosin (H&E) and differentiated in acid alcohol. After staining, sections were washed in dH₂O, dehydrated through an ethanol series, cleared in xylene and coverslipped with DPX mounting medium.

Organ Culture

SMGs were dissected in complete culture medium and reserved in pairs to compare control and treated glands from the same embryo. 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% CO₂ incubator, photographed and the medium changed daily. Skin explant cultures to confirm recombinant EDA-A1 functionality were performed as described in Mou et al. (2006).

Morphometric Analysis

The Spooner ratio (number of branches present at × hr/number of branches present at 0 hr) (Spooner and Bassett, 1989) was calculated for each SMG by manually counting branch number using an image of the explant in WCIF ImageJ 1.37c. The Spooner ratios of treated and untreated explants from the same embryo were compared by paired *t*-test, and mean Spooner ratios for groups of explants were compared by unpaired *t*-test. Two-dimensional surface areas were calculated using an image of the explant in Image Pro 6.2: the polygon tool was used to draw carefully around the epithelial portion of the SMG (excluding main duct) and scale bars used to convert the surface area in pixels of the resultant shape to mm². Data were analyzed and converted to charts using Microsoft Office Excel 2007. Box plots were constructed using Graphpad Prism 5. Statistically, the level of significance was taken as *P* < 0.05.

ACKNOWLEDGMENTS

We thank Pascal Schneider for providing the Fc EDA A1 used during experiments at the Roslin Institute, and Mike McGrew for the use of a microscope and camera. Thanks also to Nisha Patel for advice on whole-mount in situ hybridization, and Livia Katonova for technical assistance. We are grateful to the National Foundation for Ectodermal Dysplasias (NFED) for their support during this project.

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