Ectodysplasin and Wnt pathways are required for salivary gland branching morphogenesis

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SUMMARY
The developing submandibular salivary gland (SMG) is a well-studied model for tissue interactions and branching morphogenesis. Its development shares similar features with other ectodermal appendages such as hair and tooth. The ectodysplasin (Eda) pathway is essential for the formation and function of several ectodermal organs. Mutations in the signaling components of the Eda pathway lead to a human syndrome known as hypohidrotic ectodermal dysplasia (HED), which is characterized by missing and malformed teeth, sparse hair and reduced sweating. Individuals with HED suffer also from dry mouth because of reduced salivary flow. In order to understand the underlying mechanism, we analyzed salivary gland development in mouse models with altered Eda pathway activities. We have found that Eda regulates growth and branching of the SMG via transcription factor NF-κB in the epithelium, and that the hedgehog pathway is an important modulator of Eda/NF-κB. We also sought to determine whether a similar reciprocal interplay between the Eda and Wnt/β-catenin pathways, which are known to operate in other skin appendages, functions in developing SMG. Surprisingly and unlike in developing hair follicles and teeth, canonical Wnt signaling activity did not colocalize with Edar/NF-κB in salivary gland epithelium. Instead, we observed high mesenchymal Wnt activity and show that ablation of mesenchymal Wnt signaling either in vitro or in vivo compromised branching morphogenesis. We also provide evidence suggesting that the effects of mesenchymal Wnt/β-catenin signaling are mediated, at least in part, through regulation of Eda expression.

KEY WORDS: Tabby, Ectodysplasin, Edar, Salivary gland, HED, Branching morphogenesis, NF-κB, Wnt, Sonic hedgehog

INTRODUCTION
Ectodysplasin belongs to the tumor necrosis factor (TNF) family of signaling molecules and its function has been shown to be vital for the formation of the ectodermal organs in vertebrates from teleost fish to mammals (Kondo et al., 2001; Laurikkala et al., 2001; Laurikkala et al., 2002). The ectodysplasin pathway includes the ligand ectodysplasin A1 (Eda-A1, hereafter Eda), the receptor Edar and the adapter molecule Edaradd; mutations in any of the genes encoding these proteins lead to a human syndrome known as hypohidrotic ectodermal dysplasia (HED; MIM 305100 and 224900), with the characteristic features being defects in the mouse models. The submandibular salivary glands (SMG) of the Eda-deficient mouse model (Tabby) have reduced weight and less granular convoluted tubules in the adult, indicating permanent dysfunction in saliva production (Blecher et al., 1983). Eda-null glands are reported to have less ductal structures, whereas Eda-overexpressing K14-Eda mice show larger lumens (Nordgarden et al., 2004) and adult mice with enhanced Edar signaling activity were reported to have more elaborately branched salivary glands (Chang et al., 2009). Another study reported that Eda-deficient SMGs are hypoplastic, whereas Edar-deficient SMGs are severely dysplastic and suggested involvement of the Eda pathway in lumen formation (Jaskoll et al., 2003). Addition of recombinant Eda protein to salivary glands in culture has been shown to increase epithelial protein composition (Lexner et al., 2007). Despite the obvious connection to human disease, very little is known about the defects and the pathogenic mechanisms of the salivary gland defects in the mouse models. The submandibular salivary glands (SMG) of the Eda deficient mouse model (Tabby) have reduced weight and less granular convoluted tubules in the adult, indicating permanent dysfunction in saliva production (Blecher et al., 1983). Eda-null glands are reported to have less ductal structures, whereas Eda-overexpressing K14-Eda mice show larger lumens (Nordgarden et al., 2004) and adult mice with enhanced Edar signaling activity were reported to have more elaborately branched salivary glands (Chang et al., 2009). Another study reported that Eda-deficient SMGs are hypoplastic, whereas Edar-deficient SMGs are severely dysplastic and suggested involvement of the Eda pathway in lumen formation (Jaskoll et al., 2003). Addition of recombinant Eda protein to salivary glands in culture has been shown to increase epithelial branching (Jaskoll et al., 2003). These few reports indicate that Eda influences SMG morphogenesis. Interestingly, Eda is expressed in the mesenchyme of the developing mouse submandibular salivary glands, whereas in all other ectodermal organs studied so far, Eda expression is confined to epithelium (Pispa et al., 2003). The receptor Edar is expressed in the epithelium of the submandibular glands as in other ectodermal appendages. Currently, information about the mechanism of Eda regulation of SMG development, as well as a detailed analysis of the branching phenotypes of Eda loss- and gain-of-function mice, is lacking.

Individuals with HED suffer from dry mouth caused by dysfunctional salivary glands and heterozygous female carriers are best identified by reduced saliva flow and altered saliva protein composition (Lexner et al., 2007). Despite the obvious connection to human disease, very little is known about the defects and the pathogenic mechanisms of the salivary gland defects in the mouse models. The submandibular salivary glands (SMG) of the Eda deficient mouse model (Tabby) have reduced weight and less granular convoluted tubules in the adult, indicating permanent dysfunction in saliva production (Blecher et al., 1983). Eda-null glands are reported to have less ductal structures, whereas Eda-overexpressing K14-Eda mice show larger lumens (Nordgarden et al., 2004) and adult mice with enhanced Edar signaling activity were reported to have more elaborately branched salivary glands (Chang et al., 2009). Another study reported that Eda-deficient SMGs are hypoplastic, whereas Edar-deficient SMGs are severely dysplastic and suggested involvement of the Eda pathway in lumen formation (Jaskoll et al., 2003). Addition of recombinant Eda protein to salivary glands in culture has been shown to increase epithelial branching (Jaskoll et al., 2003). These few reports indicate that Eda influences SMG morphogenesis. Interestingly, Eda is expressed in the mesenchyme of the developing mouse submandibular salivary glands, whereas in all other ectodermal organs studied so far, Eda expression is confined to epithelium (Pispa et al., 2003). The receptor Edar is expressed in the epithelium of the submandibular glands as in other ectodermal appendages. Currently, information about the mechanism of Eda regulation of SMG development, as well as a detailed analysis of the branching phenotypes of Eda loss- and gain-of-function mice, is lacking.

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The development of the submandibular gland in the mouse embryo begins at ~E12, when the epithelium invaginates into the underlying mesenchyme and forms a bud (Patel et al., 2006; Tucker, 2007). The first signs of epithelial branching morphogenesis are detected at E13, when clefts are formed in the growing epithelial bud, finally giving rise to multiple separate buds. These buds thereafter branch continuously throughout the prenatal development and early postnatal development, but the most intense branching is detected between E13 and E15. The differentiation of the ducts starts at E14.5-E15, when the first columnar epithelial cells appear in the proximal end of the main duct. Thereafter, the epithelium differentiates into saliva-producing terminal buds (acini) and transporting ducts (Borghese, 1950).

The factors regulating SMG morphogenesis have been studied intensively during past 10 years (Patel et al., 2006; Tucker, 2007). The roles of the fibroblast growth factor (Fgf) family members, including Fgf1, Fgf3, Fgf7, Fgf8 and Fgf10 and their receptors, as well the function of the epidermal growth factor (Egf) pathway have been well characterized in submandibular salivary gland development (Kashima and Gresik, 1997; Hoffman et al., 2002; Koyama et al., 2008; Häärä et al., 2009). Fgf proteins and Egf family ligands act in concert with extracellular matrix and basement membrane components, such as fibronectin, collagen IV, matrix metalloproteinases and laminins (Sakai et al., 2003; Larsen et al., 2006; Tucker, 2007).

Much less is known about the roles of the other conserved signal families that regulate the development of other ectodermal organs. In vitro studies have suggested that sonic hedgehog (Shh) signaling regulates epithelial duct formation in salivary glands (Hashizume and Hieda, 2006) and also indicated involvement of Shh in branching morphogenesis (Jaskoll et al., 2004a). One of the key players in ectodermal organ formation is the Wnt/β-catenin (hereafter Wnt/β-cat) pathway. The canonical Wnt pathway was recently implicated in postnatal salivary gland development and regeneration (Hai et al., 2010), but practically nothing is known about its role during embryonic salivary gland development. The role of Eda has been addressed in few studies that have indicated the stimulatory effect of Eda signaling on salivary gland development (see above). However, the pathogenesis behind the salivary gland defects associated with HED, and the intracellular mediators and downstream targets of Eda, have not been characterized in detail.

To address the molecular mechanisms that lead to impaired function of salivary glands in individuals with HED, we analyzed submandibular salivary gland development in Eda mutant mice, both loss- and gain-of-function, followed by in vitro morphometrical and functional studies. We report that branching is highly dependent on Eda activity and that NF-xB is an essential mediator of Eda signaling. We provide evidence to indicate that Shh is a crucial downstream component of Eda/Edar/NF-xB pathway in the salivary gland and is required to mediate Eda-induced branching morphogenesis. Our data also reveal that suppression of mesenchymal Wnt/β-cat signaling leads to decreased SMG branching morphogenesis accompanied by downregulation of Eda expression.

**MATERIALS AND METHODS**

**Animals**
The following mouse strains and their genotyping have been described earlier: K14–Eda (Mustonen et al., 2003), Eda deficient (Tabby) (Pispa et al., 1999), IcbΔαAN (Schmidt-Ullrich et al., 2001) and NF-xB REP mice (Bhakar et al., 2002). Tabby mice were of CBAB6 background, K14-Eda mice were of FVB background and NF-xB REP of C57Bl/6 background. NF-xB REP mice were bred into Eda-/- and K14-Eda backgrounds to monitor NF-xB activity. IcbΔαAN mice were in a C57Bl/6 background, and were crossed with NF-xB REP mice for the analysis of NF-xB reporter expression. Batgalf mice (Maretto et al., 2003) were kindly provided by Stefano Piccolo (University of Padua, Italy) and maintained in C57Bl/6; Topgal mice (DasGupta and Fuchs, 1999) and B6;129Sgt(ROSA)26Sor/J (Rosa 26) mice were from Jackson Laboratories; Axin2/ConductinlacZ/Eda2z (Axin2lacZ/Eda2z) mice (Lustig et al., 2002) were kindly provided by Walter Birchmeier (MDC, Berlin, Germany). Heterozygous Shh-CreGFP mice (Harfe et al., 2004) expressing green fluorescent protein under Shh promoter, were maintained in NMRI background. To obtain DermoCre-/+; β-cat-/- mice, male mice of the genotype DermoCre (neo-); β-catlacZ/− (Huelsken et al., 2000) were crossed with female mice of the genotype β-catlacZ/− (Huelsken et al., 2001). The FRT-neo cassette of the DermoCre mice (Yu et al., 2003) had been removed using the FLPeR mice (Farley et al., 2000). The appearance of a vaginal plug was taken as embryonic day (E) 0, and embryos were carefully staged according to limb morphogenesis.

**Organ cultures**
Submandibular/sublingual salivary gland rudiments (referred to as SMGs) were cultivated in Trowell-type culture as described previously (Flinnia et al., 2008; Hääri et al., 2009). In short, the explants were cultured in air-liquid interface on filters (Whatman Nucleapore, 0.1 μm) in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (PAA laboratories, Pasching, Austria), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For cultures longer than 2 days, DMEM and F12 medium (1:1) was used and supplemented with 10% fetal calf serum, ascorbic acid (0.075 g/l), glutamine and penicillin-streptomycin. The following proteins or inhibitors were used at concentrations indicated in the text: Fc-Eda-A1 (Gaide and Schneider, 2003), sonic hedgehog (R&D Systems), CKI-7 (Sigma-Aldrich), cyclopamine (Sigma-Aldrich) and XAV939 (Stemgent). For microscopy and morphometric analyses, the samples were imaged under Olympus SZX9 stereomicroscope or under Olympus AX70 microscope. Data were analyzed by freely available ImageJ software (NCBI). All statistical comparisons were made between mutants and their littermate controls.

**Histology**
The tissues were fixed in 4% paraformaldehyde and taken through ethanol and xylene and paraffin and sectioned at 7 μm for histology. The size of the salivary gland epithelium of DermoCre-/+; β-cat-/- mice and their littermate controls was measured from serial tissue sections: the area of the glands was calculated from the number of sections times the thickness of the sections was carried out according to standard protocols using 35S-UTP labeling (Mannheim Gmbh, Germany). Radioactive in situ hybridization on paraffin sections was detected with BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim GmbH, Germany). Histology

In situ hybridization

The embryonic tissues were fixed in 4% paraformaldehyde (PFA) for 24 hours in +4°C and dehydrated through methanol series (25, 50, 75, 100%). Whole-mount in situ hybridization was performed with InSituPro robot (Intavis AG, Germany) and section in situ hybridization as previously described (Flinnia et al., 2008) using probes specific to Shh, Dhh, Ptc1, Eda and Edar (Bitgood and McMahon, 1995; Laurikkala et al., 2002). The quantification of Eda expression was carried out using ImageJ from dark-field images (threshold=200), measuring the mean luminosity in the mesenchyme in each gland.
X-gal staining
Embryos were fixed for 30 minutes in 2% paraformaldehyde, 0.2% glutaraldehyde, washed three times for 10 minutes in Dulbecco’s PBS (pH 7.5) containing 2 mM MgCl₂ and 0.02% Nonidet P-40. Samples were stained overnight at room temperature with X-gal staining solution [1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ and 10% Nonidet P-40 in PBS (pH 7.5)]. Whole-mount stained tissues were post-fixed in 4% PFA overnight at +4°C, embedded in paraffin and sectioned at 7 µm.

Quantitative PCR
Dissected salivary glands were placed into 350 µl lysis buffer of the RNeasy mini kit (Qiagen) containing 1% β-mercaptoethanol (Sigma). Total RNA was isolated as specified according to the manufacturer’s instructions and quantified using a nanodrop spectrophotometer. A minimum of eight samples were analyzed in each experiment. 100 ng of total RNA was reverse transcribed using 500 ng of random hexamers (Promega) and 100 units of Superscript II (Invitrogen) following the manufacturer’s instructions. Lightcycler DNA Master SYBR Green I (Roche) was used with a Lightcycler 480, and the software provided by the manufacturer was used for analysis. The data were normalized against Ranbp gene (Ranbp1 – Mouse Genome Informatics) (Fliniaux et al., 2008). Primer sequences are available upon request. Gene expression was quantified by comparing the sample data against a dilution series of PCR products of the gene of interest.

RESULTS
Branching depends on, and correlates with, Eda activity
To study the effect of Eda signaling on branching morphogenesis of the salivary glands, we focused on the early stages of branching at E13-E15, and used a well-established ex vivo culture system for submandibular salivary glands (SMG), which is known to recapitulate the branching pattern that occurs in vivo and allows an accurate quantification of forming branches (Borghese, 1950; Patel et al., 2006). SMG development was compared between Eda loss-of-function (Eda deficient, Tabby), wild-type and Eda gain-of-function (K14-Eda) mice. The SMG rudiments were dissected at E13 and cultured for 2 days. The glands of Eda−/− mice were similar to wild type in appearance at the onset of branching, at E12.5-E13. However, after 2 days of culture, the epithelium was less branched and smaller, with significantly fewer (~65%) end buds compared with wild type (Fig. 1A,B,D). Overexpression of Eda under the K14 promoter led to a substantial increase in branching and end bud formation (Fig. 1C,D).

Shh is a crucial target of Eda in the salivary gland
Shh has been shown to be involved in SMG development (Jaskoll et al., 2004a), and there is evidence that Shh may be a direct downstream target of Eda in hair follicles (Cui et al., 2006; Schmidt-Ullrich et al., 2006; Pummila et al., 2007). Therefore, we analyzed the connection between Eda and Shh in the developing salivary gland. Quantification of Shh transcripts in Eda null, control and K14-Eda transgenic SMGs at E14 revealed that the amount of Shh correlated with the Eda status (Fig. 2A). However, Shh expression was not completely absent in Eda−/− glands, indicating that other factors besides Eda are likely to regulate Shh expression. Supplementation of Shh protein to cultured E13 and E14 SMG was previously shown to increase branching morphogenesis, whereas hedgehog pathway antagonist cyclopamine suppressed branching (Jaskoll et al., 2004a). To test whether the effect of Shh signaling depends on the level of Eda expression, we treated E13 Eda−/−, control and K14-Eda salivary glands with recombinant Shh protein (500 ng/ml). We observed a prominent increase in branching in Eda-null explants, a significantly weaker response in control and no apparent effect in K14-Eda SMGs (Fig. 2B). Next, we compared the ability of Shh and recombinant Eda protein (Fc-Eda 500 ng/ml) (Gaide and Schneider, 2003) to restore the Eda−/− phenotype. Both Fe-Eda and Shh induced a similar, about 1.4-fold, increase in the number of end buds (Fig. 2C-F). The rescue was not complete though, as the average number of end buds after 2 days culture was less than in wild-type glands (see Fig. S1 in the supplementary material). By contrast, treatment of E13 Shh−/− salivary glands, which were less branched than wild type, for 2 days with Eda protein, had no significant effect, yet Shh protein rescued branching as expected (see Fig. S2 in the supplementary material).

To test whether changes caused by differences in Eda expression levels were affected by suppression of hedgehog signaling, cyclopamine (5 µM) was added to salivary glands dissected from E13 Eda−/−, control and K14-Eda embryos. Cyclopamine reversed excessive branching in K14-Eda glands significantly and reduced branching clearly in control glands, but had no effect on Eda−/− glands (Fig. 2G-M). Thus, inhibition of hedgehog signaling by cyclopamine in wild-type and K14-Eda salivary glands phenocopied Eda−/− salivary glands. Taken together, the effect of both enhancing and inhibiting hedgehog pathway was strictly dependent on Eda signaling activity.

If Shh was a downstream target of Eda in the developing salivary gland, Shh transcripts should be localized in the epithelium where Edar expression and signaling activity is confined (Pispa et al., 2003) (see also below). Radioactive in situ hybridization with a Shh-specific probe revealed low level of Shh expression in the epithelium of wild-type salivary glands, which was augmented in K14-Eda glands, but was similar to negative control in Eda−/− glands (Fig. 3A-C). In addition, Ptc1, a transcriptional target of Shh, was expressed at low levels in developing SMGs (see Fig. S2 in the supplementary material). To address whether other hedgehog
In developing teeth and hair follicles, the effects of Eda are mediated largely by transcription factor nuclear factor-κB (NF-κB) (Schmidt-Ullrich et al., 2001; Pispa et al., 2008). Inhibition of NF-κB in vitro by cell-permeable peptide SN50 was reported to inhibit SMG branching morphogenesis (Melnick et al., 2001a). However, more recent studies by the same group (Melnick et al., 2009) suggested that NF-κB activity is not essential for Eda signaling in the salivary gland. To study this obvious discrepancy, we first analyzed NF-κB activity with a NF-κB lacZ reporter mouse (Bhakar et al., 2002; Pispa et al., 2008) in the developing salivary gland. At all stages studied (E12-E17), we observed strong NF-κB signaling activity in the epithelium (Fig. 4A-E; see Fig. S3 in the supplementary material), where also Edar was expressed (Fig. 4F,G) (Pispa et al., 2003). However, NF-κB activity was not distributed equally throughout the epithelium, but was more intense in the outermost cell layer (Fig. 4B-E) adjacent to the mesenchymal source of Eda (Pispa et al., 2003).

Next, we analyzed the NF-κB reporter activity in the Eda−/− background. We did not detect NF-κB activity in Eda-null salivary glands at any stage studied (E12-E17) (Fig. 4I; and data not shown), indicating that NF-κB signaling in SMG is entirely dependent on Eda. Furthermore, increased NF-κB reporter activity was observed in K14-Eda transgenic embryos compared with their control littermates (Fig. 4J; and data not shown). To study whether Eda can induce NF-κB activity in vitro, we treated Eda−/−; NF-κB reporter glands with different concentrations of Eda protein and followed the expression of the reporter. After 1 day of treatment, NF-κB activity appeared dose dependently in Eda−/− salivary glands (Fig. 4K-M). However, even at the highest tested concentration of Fe-Eda, the NF-κB activity was lower than in wild-type glands cultured under the same conditions (Fig. 4N), and was limited to the margins of the epithelium more strictly than in control glands. This suggests that the recombinant protein had a limited ability to penetrate through the salivary tissue ex vivo. This finding may also explains the incomplete rescue of Eda−/− salivary glands by the Eda protein (Fig. 2C; see Fig. S1 in the supplementary material).
In order to study the importance of NF-κB signaling in salivary gland branching morphogenesis, we analyzed IκBαΔN mice, which lack NF-κB activity (Schmidt-Ullrich et al., 2001). Suppression of NF-κB signaling in these mice is achieved by ubiquitous expression of the transdominant super-repressor IκBαΔN. Analysis of NF-κB reporter expression confirmed that NF-κB activity was completely inhibited in SMGs of IκBαΔN embryos (Fig. 5A-C). Importantly, loss of NF-κB activity led to a similar branching defect as seen in Eda–/– mice (Fig. 5D,E,H; compare to Fig. 1). To test whether overexpression of Eda can induce branching independently of NF-κB activity, we crossed IκBαΔN mice with K14–Eda mice and analyzed the branching pattern of cultured SMGs. IκBαΔN and compound IκBαΔN; K14–Eda mutants showed a similar branching defect, with reduced branching compared with wild-type and K14–Eda littermates (Fig. 5D-H). These findings show that Eda-induced branching is completely dependent on NF-κB in developing salivary glands.

**Wnt activity is restricted to the mesenchyme during early stages of salivary gland development**

In many ectodermal appendages, canonical Wnt activity colocalizes with Edar/NF-κB activity in the epithelium and studies on hair follicles have shown that Wnt and Eda pathways regulate partially the same epithelial target genes (Flinaiaux et al., 2008; Zhang et al., 2009). Moreover, in hair follicles Wnt/β-catenin pathway has been placed upstream of Edar, which in turn is thought to regulate expression of Wnt ligands (Zhang et al., 2009). To address whether a similar interplay between the two pathways operates also in developing salivary glands, we analyzed the localization of Wnt activity using the Axin2–Gal reporter mice (Lustig et al., 2002). At E13-E14, Axin2–lacZ expression was entirely confined to the mesenchyme surrounding the branching epithelium and was strikingly different from the NF-κB reporter expression. Scale bars: 500 μm for A,B,D,F,H–N; 100 μm for C,E,G.
E13 to E15 either (see Fig. S4 in the supplementary material). Thus, the complementary activities of NF-κB and Axin2 reporters in epithelium and mesenchyme, respectively, suggest that the situation in salivary glands may differ from other ectodermal appendages.

**Inhibition of mesenchymal Wnt/β-catenin signaling impairs SMG branching morphogenesis**

To assess the significance of mesenchymal Wnt signaling in the early branching morphogenesis of SMG, we analyzed the effect of Wnt inhibition in vitro and in vivo. First, we treated E13 wild-type salivary glands for 2 days with the Wnt signaling inhibitors XAV939 (Huang et al., 2009) or CKI-7 (Peters et al., 1999; Fliniaux et al., 2008) (Fig. 7A-D). As the reporter studies indicated that there was no canonical Wnt signaling in the epithelium at these stages, these inhibitors were expected to affect mesenchymal Wnt activity only. Twenty-four hour treatment with XAV939 (10 μM or 100 μM) did not have any obvious effect, yet 2 day treatment led to a significant reduction in epithelial branching and growth (Fig. 7E), which resembled the Eda-null and IκBαΔN mutant phenotypes. Under these conditions, XAV939 inhibited Wnt signaling, visualized by reduced lacZ expression in Axin2-lacZ SMGs (see Fig. S5 in the supplementary material). In addition, treatment with 100 μM CKI-7 caused a decrease in the number of end buds (Fig. 7F). To validate these in vitro findings, we analyzed conditional β-catenin-null mice, DermoCre+; β-cat−/− mice, in which β-catenin is deleted using Cre expressed in the mesenchyme. Analysis of lacZ expression in DermoCre mice crossed with the Rosa26 reporter mice confirmed widespread mesenchymal Cre activity in SMG at E14 (data not shown). SMGs of DermoCre+; β-cat−/− were analyzed at E13 to E15, and showed a significantly smaller size and reduced branching as compared with control littermates (Fig. 7G-I). Collectively, these results imply that mesenchymal Wnt signaling is required for the growth and branching of the submandibular salivary gland. The identity of the Wnt ligand(s) regulating SMG morphogenesis is currently unclear, but we detected Wnt4 and Wnt6 initially in the mesenchyme (E13), and 1 day later in the epithelium. In addition, Wnt11 was expressed in the mesenchyme (see Fig. S4 in the supplementary material).

Lef1, a transcription factor mediating Wnt/β-catenin signaling, has been suggested to regulate the expression of Eda, and a conserved Lef1-binding site is found in the promotor region of Eda (Durmowicz et al., 2002; Laurikkala et al., 2001; Srivastava et al., 1997); therefore, we tested whether mesenchymal expression of Eda in developing SMG was affected by inhibition of mesenchymal Wnt signaling. Twenty-four hour treatment of E13 SMGs with 100 μM XAV939 led to a notable, but statistically non-significant, reduction in the amount of Eda mRNA (data not shown). Importantly, in situ hybridization revealed that expression of Eda was downregulated in vivo in salivary glands of DermoCre+; β-cat−/− embryos compared with their control littermates (Fig. 7J-L). These findings led us to hypothesize that Eda could be an important downstream mediator of the Wnt/β-catenin pathway in developing SMGs. If this was the case, exogenous Eda protein should overcome the branching defect caused by suppressed Wnt/β-catenin activity. To test this, we cultured control and XAV939-treated E13 salivary glands for 2 days in the presence of recombinant Eda protein (Fig. 7M-P). We observed that Fc-Eda had a significant restoring effect on branching of XAV939-treated SMG explants, the number of end buds being normalized close that of control explants (Fig. 7O-Q). Moreover, the ability of Eda to stimulate branching was substantially higher in XAV939-treated glands compared with control glands (Fig. 7R).
DISCUSSION

Owing to the apparent phenotypic similarity of Eda mutant (Tabby) mice and humans carrying inactivating mutations in Eda, the mouse models have turned out to be highly useful tools with which to unravel the molecular pathogenesis of HED. Thus far, the glandular aspects of HED have not been studied intensively, although over 20 different exocrine glands are known to be affected in Eda mutant mice and/or individuals with HED (Clarke et al., 1987; Grüneberg, 1971). In general, the glands are either missing or hypoplastic. The first case is exemplified by sweat glands, tracheal submucosal glands and minor salivary glands where a lack of the initial outgrowth leads to organ agenesis (Grüneberg, 1971; Rawlins and Hogan, 2005; Kunisada et al., 2009; Wells et al., 2011). For most glandular organs, however, the Eda pathway appears dispensable for initiation, but is required either for epithelial growth, branching and/or cytodifferentiation (Grüneberg, 1971). Here, we report a comprehensive morphological and molecular analysis of salivary gland development in mice mutant for the Eda/NF-κB pathway using the ex vivo SMG explant culture system. We show that loss of Eda compromised SMG growth and branching, whereas excess of Eda led to highly accelerated branching morphogenesis. We propose that the diminished branching of embryonic Eda-null glands eventually leads to smaller salivary glands with reduced epithelial surface area resulting in decreased saliva production seen in individuals with HED. In addition, the saliva composition is altered in individuals with HED (Lexner et al., 2007), but it is currently unclear whether this reflects a functional defect in mature salivary glands or whether the development of distinct salivary glands (sublingual, submandibular and parotid), which are known to produce unequal types of saliva (Tucker, 2007), is differentially affected by the lack of Eda.

Our analysis of mice with suppressed NF-κB signaling activity, singly and in combination with mice overexpressing Eda, conclusively demonstrated the necessity of Eda/Edar/NF-κB for SMG morphogenesis. Furthermore, the ability of Eda to regulate salivary gland development relied on intact NF-κB activity. Analysis of NF-κB reporter mice revealed that activation of NF-κB is entirely dependent on Eda during embryonic SMG development and not, for example, on TNFα, as recently suggested (Melnick et al., 2009). Previous studies have shown that supplementation of TNFα substantially increases epithelial cell proliferation and branching of cultured SMGs (Melnick et al., 2001b), and in light of our findings it is possible that as a powerful inducer of NF-κB, exogenous TNFα may have mimicked the effect of Eda, as previously shown in embryonic Eda−/− skin explants where TNFα rescued primary hair placode formation (Schmidt-Ullrich et al., 2006). Contrary to previous speculations (Jaskoll et al., 2003), analysis of the NF-κB reporter mice indicated that the Eda/NF-κB pathway is active already at the earliest developmental stages, although our data show that it is dispensable for initiation.

Fig. 6. Wnt reporter activity does not colocalize with NF-κB activity at any stage. (A-D′) Wnt/β-cate activity in developing SMGs was assessed using Axin2lacZ/+ embryos. (A-B′) β-Gal activity localized to the mesenchyme at E13-E14; compare with NF-κB reporter expression in Fig. 4A-E. (C-C′) At E15, Axin2-lacZ expression was detected both in the mesenchyme and the main epithelial duct. (D-D′) At E16 Axin2-lacZ was solely observed in developing ducts. Black arrows highlight Axin2-lacZ expression, white arrows its absence. (E-H) Comparison of Axin2-lacZ and the NF-κB reporter activities in wholemounts revealed non-overlapping expression at E15 and E16. At E16, the ducts (broken line in H) were positive for Wnt but not for NF-κB reporter, whereas the developing alveoli were positive for NF-κB but not for Wnt activity. Scale bars: 500 μm for A-D and A′-D′; 100 μm for A″-D″.
of SMG morphogenesis. The similarity of the SMG phenotypes of Eda-null and kBΔN mutant mice and the absence of any residual NF-κB signaling activity in both mutants demonstrate that lack of an early phenotype is not due to redundancy with another pathway activating NF-κB.

**Shh is an important mediator of Eda signaling in the developing salivary gland**

Downstream target genes of the Edar/NF-κB pathway have been under intensive study during recent years. Shh, Wnt10a and Wnt10b, Wnt pathway inhibitor Dkk4, and BMP inhibitors Ccn2/Ctgf (CCN family protein 2/connexin growth factor) and follistatin have been shown to be downstream of the Eda pathway in hair and/or tooth development (Cui et al., 2006; Schmidt-Ullrich et al., 2006; Pummila et al., 2007; Fliniaux et al., 2008; Zhang et al., 2009). Of the confirmed or suspected transcriptional targets of Eda, Shh is so far the only one studied in the context of salivary gland development (Jaskoll et al., 2004a).

Here, we report that the response of cultured SMGs to the manipulation of the hedgehog pathway, either by application of Shh is an important mediator of Eda signaling in the developing salivary gland

**Fig. 7. Suppression of mesenchymal Wnt/β-catenin activity reduces branching and size of the SMG.** (A-D) E13 SMGs were cultured for 2 days in the presence of tankyrase inhibitor XAV939 or casein kinase inhibitor CKI-7. XAV939 [10 μM (n=16) and 100 μM (n=6)] reduced branching by 40% compared with controls; 100 μM CKI-7 also had a similar effect. (E) Mean number of end buds±s.e.m. of SMGs cultured for 1 or 2 days in the presence of XAV939. (F) Mean number of end buds±s.e.m. of SMGs cultured for 2 days in the presence of CKI-7. (G-H) Histology of the salivary glands of DermoCre+; β-cat–/fl embryos at E14. Deletion of mesenchymal β-catenin led to an overall smaller size and fewer branches compared with control littermates. Scale bars: 500 μm. (I) Quantification of the epithelial size and the number of end buds from serial sections in DermoCre+, β-cat–/fl embryos at E13 to E15 (n=3 for E13; n=6 for E14, and n=3 for E15) in comparison with control littermates. Values for control SMGs were set at 1 (broken line). Data are mean±s.e.m. (J-K) Radioactive in situ hybridization showed reduced expression of Eda in DermoCre+, β-cat–/fl salivary glands compared with control littermates at E14. (L) In situ hybridization signal of mesenchymal Eda expression was quantified in E14 DermoCre+, β-cat–/fl (n=4) and control salivary glands (n=4), four sections per sample were analyzed. Data are mean±s.e.m. (M-P) E13 salivary glands were cultured for 2 days in the presence or absence of 500 ng/ml of recombinant Eda and 10 μM XAV939. Eda protein restored the XAV939-induced branching defect (n≥6 in each group). (Q) Mean number of end buds±s.e.m. of data shown in M-P. (R) Response to Eda (fold increase in number of end buds) was significantly higher in XAV939-treated (n=15) salivary glands compared with untreated (n=6) ones. Data are mean±s.e.m. ***P<0.001, **P<0.01, *P<0.05.
The role of Wnt/β-catenin signaling in branching morphogenesis has not been studied in detail in cutaneous glands, with the exception of mammary glands (for a review, see Boras-Granic and Wysolmerski, 2008). High epithelial Wnt reporter activity is seen during onset of embryonic branching morphogenesis (Chu et al., 2004), and suppression of canonical Wnt activity via deletion of the co-receptor Lrp6 compromises branching development (Lindvall et al., 2009). In developing salivary glands, we did not detect Wnt pathway activity in branching buds or alveoli with any of the three reporters analyzed. The reporter studies seem to suggest that epithelial Wnt signaling activity does not regulate branching morphogenesis in the SMGs, but may instead be involved in ductal differentiation and lumen formation.

Numerous studies have revealed the importance of epithelial-mesenchymal interactions in salivary gland development, and classic tissue recombination experiments suggest that the branching pattern is controlled largely by signals from the mesenchyme (Patel et al., 2006; Tucker, 2007). Our results indicate that mesenchymal Wnt/β-catenin activity regulates SMG morphogenesis. Inhibition of Wnt signaling either in vivo or in vitro produced smaller glands with fewer end buds. This function may be transient though, as mesenchymal expression of Axin2-lacZ reporter vanished by E16. The mechanism for how mesenchymal Wnt/β-catenin signaling regulates epithelial growth and branching has yet to be clarified, but the Wnt target genes may include paracrine factors such as some of the many Fgfs (Hoffman et al., 2002; Patel et al., 2006; Steinberg et al., 2005). Our data suggest that transcriptional regulation of Eda may be an important function of mesenchymal Wnt/β-catenin signaling, and that Eda in turn acts as a mesenchymal cue to regulate epithelial branching via NF-κB and Shh (Fig. 8).

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
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**References**

**Fig. 8. Regulation of salivary gland morphogenesis by Eda signaling and its integration with Wnt and Shh pathways.** Eda expression is restricted to the SMG mesenchyme and is downstream of Wnt/β-catenin signaling. Eda is synthesized as an integral membrane protein and its cleavage is required for biological activity. Eda translocates to the epithelial compartment and binds to its receptor Edar, which ultimately leads to the activation of the transcription factor NF-κB. The degradation of the repressor IκBα promotes the transport of NF-κB to the nucleus where it induces the expression of Shh and other target genes essential for epithelial growth and branching.

It is conceivable that Shh is not the only important target gene of the Eda pathway in the salivary gland. Besides Shh, exogenous Eda protein induces the expression of follistatin in cultured embryonic skin explants (Pummila et al., 2007). Although the function of endogenous follistatin in salivary gland development is unknown, it may have a role as an antagonist of activin, which causes a severe disruption of SMG morphogenesis when applied ex vivo (Ritvos et al., 1995). With increasing knowledge of the downstream events of the Eda pathway (Mikkola, 2009), novel target genes and their functional relevance are likely to be uncovered and will be tested in the future.

**Mesenchymal Wnt signaling is required for Eda expression and SMG branching morphogenesis**
Zhang et al. (Zhang et al., 2009) recently showed a sequential interdependency between the Wnt and Eda pathways in developing hair follicles: Wnt/β-catenin signaling is essential for NF-κB activation whereas Edar/NF-κB is thereafter required to strengthen and maintain Wnt/β-catenin activity. The link between the two pathways is further supported by the conspicuous similarities of individuals carrying mutations in Eda/Edar/Edaradd or in Wnt10a (Adaimy et al., 2007; Cluzeau et al., 2011) (OMIM 257980), which is one of the proposed targets of Edar/NF-κB (Zhang et al., 2009). Moreover, the transcriptional targets of the two pathways are partially overlapping (Fiiniaux et al., 2008; Zhang et al., 2009), and in developing hair follicle, tooth and mammary buds, high Wnt/β-catenin and NF-κB signaling activities colocalize in the epithelium (Chu et al., 2004; Liu et al., 2008; Pipsa et al., 2008; Zhang et al., 2009). Surprisingly, we did not find a similar correlation between Wnt and NF-κB reporter expression patterns in developing salivary glands, suggesting that unlike in hair follicles, Wnt/β-catenin and Eda/NF-κB may not collaborate in regulation of target gene expression in SMG.

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