

Intercellular Growth Factor Signaling and the Development of Mouse Tracheal Submucosal Glands

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To provide a genetic framework for investigating changes in airway submucosal gland function in human respiratory disease, we have investigated their counterparts in normal and mutant mice. We describe their morphogenesis in relation to the expression of genes encoding conserved intercellular signaling pathways. Submucosal glands are severely reduced in number and size in mice heterozygous for *Fgf10*. Glands are completely absent in mice lacking Ectodysplasin (*Eda*) and Edaradd (*Eda* receptor adaptor protein), members of the tumor necrosis (TNF) superfamily of signaling factors. Furthermore, components of the *Eda* and closely related pathways are transcribed throughout the respiratory system in the adult mouse. Finally, the temporal and spatial pattern of *Bmp4* expression suggests that it may control submucosal gland development and homeostasis. Taken together, our observations have important implications for the better understanding of the submucosal gland remodeling that occurs in human respiratory disease. *Developmental Dynamics* 233:1378–1385, 2005. © 2005 Wiley-Liss, Inc.

Key words: submucosal gland; trachea; mouse; FGF; BMP; Ectodysplasin

Received 15 March 2005; Revised 6 April 2005; Accepted 10 April 2005

INTRODUCTION

The submucosal glands (SMGs) of the upper respiratory tract play an important role in secreting mucus, lysozyme, defensins, and other agents that help protect the lungs from particles and infectious agents. They develop from small buds that arise in the dorsolateral airway epithelium between the cartilage rings. These buds extend into the surrounding mesenchyme, undergo branching morphogenesis, and differentiate into mucous and serous cells that produce distinct secreted products (Finkbeiner, 1999). In the human, SMGs are found along the airways from the larynx down to the distal part of the main bronchi. However, in the mouse, they are re-

stricted to the upper trachea, more specifically to the regions between the first few cartilage rings, with the precise distribution depending on genetic background (Borthwick et al., 1999; Innes and Dorin, 2001). The significance of the SMGs for human health lies in the fact that they undergo enlargement in patients suffering from respiratory disorders. These disorders include severe asthma and bronchitis, both associated with airway inflammation and the secretion of excessive amounts of mucus (Jeffery, 2000). For example, in patients with severe/fatal asthma, the size of SMGs increases more than twofold as judged from histological sections of bronchial biopsy and postmortem material (Benayoun et al., 2003). Hypertro-

phy and hyperplasia of SMGs are also seen in dogs and rats exposed to environmental agents such as sulfur dioxide that damage the surface epithelium (Lamb and Reid, 1968). Furthermore, recent work suggests that a change in the volume and the viscosity of secretions from the SMGs may be the primary defect in cystic fibrosis (Salinas et al., 2004). However, despite the significance of SMGs for human respiratory disease, little is known about the mechanisms controlling their growth, differentiation, and homeostasis, either in the early postnatal animal or in the adult. It is likely that these mechanisms are activated or subverted in response to conditions that ultimately cause pathological changes in SMGs.

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Grant sponsor: NIH; Grant number HL080517.

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DOI 10.1002/dvdy.20461

Published online 22 June 2005 in Wiley InterScience (www.interscience.wiley.com).

One approach to identifying factors controlling SMG growth and differentiation is to study mouse mutants in which their development is defective. Along these lines, it was shown several years ago that SMGs are absent from *Lef1* null mutant mice, suggesting that the canonical Wnt signaling pathway is involved in their development (Duan et al., 1999). The localization of *Lef1* transcripts to the early buds of the neonatal ferret tracheal SMGs is consistent with the initiation of the glands being dependent on a Wnt signal from either the underlying mesenchyme or adjacent epithelium (Duan et al., 1998; Driskell et al., 2004). *Lef1* is also required for the development of many ectodermal appendages, including hair follicles, whiskers, teeth, and mammary gland (van Genderen et al., 1994; Das-Gupta and Fuchs, 1999; Sasaki et al., 2005). In all cases, there is a dynamic and reciprocal interaction between the mesenchymal and epithelial components, mediated by signaling factors that include not only the Wnts but also bone morphogenetic proteins (Bmps), fibroblast growth factors (Fgfs), Sonic hedgehog, and their respective antagonists. The potential role of these factors in the induction of SMGs and their subsequent morphogenesis has not been explored.

In this study, we follow-up on the original observation by Gruneberg that tracheal SMGs are absent in *Tabby* mice. These mutants also have defects in the development of hair, teeth, and sebaceous and salivary glands (Gruneberg, 1971a,b). *Tabby* is an X-linked gene that encodes *Ectodysplasin* (*Eda*), a member of the TNF superfamily of intercellular signaling factors (Ferguson et al., 1997; Srivastava et al., 1997; Mikkola and Thesleff, 2003). A similar phenotype is seen in mice with mutations in autosomal genes encoding components of the *Eda* signaling pathway. These genes include *Edar*, which is mutated in *downless* (*Edar^{dl}*) mice and encodes a TNF receptor of the death domain class, and *Edaradd*, which is mutated in *crinkled* (*Edaradd^{cr}*) mice and encodes an adaptor protein with a death domain (Headon and Overbeek, 1999; Headon et al., 2001; Thesleff and Mikkola, 2002). The *Eda/Edar* signaling pathway is evolutionarily conserved and mutations in the corre-

sponding human genes, *EDA*, *EDAR*, and *EDARADD*, cause the inherited disorder known as hypohidrotic ectodermal dysplasia (HED). Patients are characterized by abnormalities in multiple organs including hair, teeth, and sweat and sebaceous glands. Significantly for this study, patients with HED lack mucus-producing glands in the nose, larynx, and bronchi and frequently have severe chronic pulmonary complications that may require lung transplantation (Smythe et al., 2000).

To learn more about the mechanisms underlying the normal and abnormal development of SMGs in the mouse, we have followed their development in relation to genes encoding signaling factors that control epithelial budding and branching morphogenesis in other organ systems such as hair, teeth, and the distal lung. These include BMPs, HHs, and FGFs. In addition, we have confirmed that SMGs are indeed absent in adult *Tabby* and homozygous mutant *crinkled* mice. Finally, we present evidence that the *Eda/Edar* and other TNF signaling pathways are expressed in the lung outside of the SMGs and, therefore, may play more extensive roles in maintaining the homeostasis of the lung and its response to pathological conditions.

RESULTS

Early Development of Normal Submucosal Glands

The buds that give rise to the SMGs first appear in the most proximal intercartilage region of the tracheal epithelium at postnatal day 2 (P2; Fig. 1A). By P4, the first buds to form have enlarged and extended and have undergone some branching, but new buds are still forming. There is evidence at this stage, but not earlier, for some production of mucus within the older glands, as judged by Alcian blue staining and transcription of the gene encoding Demilune Cell and Parotid Protein (*Dcpp*), which is produced by the mature glands (Fig. 1B, and data not shown). In addition, immunohistochemistry with anti-smooth muscle actin reveals myofibroblasts around the developing glands at this time (Fig. 1D). Mucus production is well under way by P7 (Fig. 1C). Even at P7 and P14, new buds are still forming and

proliferation of cells within these buds can be detected by a short pulse of labeling with bromodeoxyuridine (BrdU; data not shown). However, no evidence for new bud formation is seen in animals older than P21. These results suggest that the factors inducing the formation of new SMGs are likely to be present over a period of several weeks.

At P2, when buds first appear, basal cells positive for keratin 14 (K14) are present within the tracheal epithelium (Fig. 1F, arrows). The initial buds, and the proximal regions of the early glands that will form the future ducts, appear to be enriched in K14-positive cells (Fig. 1F, arrowheads). K14 basal cells are also present within the ducts and secretory region of P7 and adult glands (Fig. 1G, arrows and data not shown), as noted by others for adult mice (Borthwick et al., 2001).

Gene Expression in the Mesenchyme Associated With Developing Submucosal Glands

The development of hair follicles in the embryonic mouse skin is associated with the dynamic and localized expression of genes encoding extracellular signaling factors within either the mesenchymal dermis or the epidermis. Among these genes are members of the *Bmp* superfamily, including *Bmp4* (Kulesa et al., 2000; Botchkarev and Sharov, 2004). We therefore examined the expression in the developing SMGs of *Bmp4^{lacZ}*, which faithfully reports gene transcription (Lawson et al., 1999). As shown in Figure 2, at P2 *Bmp4^{lacZ}* is widely expressed in the mesenchyme underneath the tracheal epithelium and around early buds. By P7 expression is more restricted to the mesenchymal cells around the developing glands and by 4 weeks after birth, *Bmp4^{lacZ}* is only seen in a few mesenchymal cells underneath the tracheal epithelium and SMG acini (Fig. 2C,D). Expression is not detected in the epithelial cells at any stage.

Fgf10 Is Required for Submucosal Gland Morphogenesis

Studies in hair and feather development have implicated Fgfs in the re-

ciprocal signaling between mesenchyme and epithelium at several stages (Ohuchi et al., 2003; Petiot et al., 2003; Mandler and Neubuser, 2004). In addition, recent results have uncovered a role for Fgf10 in the development of the lacrimal and salivary

glands of both the mouse and human. Fgf10 is transcribed in the mesenchyme adjacent to several different budding organs, these organs include the lacrimal gland, feather placodes, and distal lung buds (Bellusci et al., 1997; Makarenkova et al., 2000; Man-

dlar and Neubuser, 2004). Fgf10 heterozygous mice do not develop lacrimal glands and have hypoplastic or absent salivary glands (Makarenkova et al., 2000; Entesarian et al., 2005). We therefore examined serial sections through the trachea of Fgf10^{+/-} heterozygotes and their wild-type siblings. At P20, when the SMGs are largely developed, wild-type mice had well-formed, highly branched glands above the first tracheal ring and smaller SMGs between the more distal cartilage rings extending as far as the sixth cartilage element (Fig. 3A,B, and data not shown). The few SMGs present in the Fgf10^{+/-} trachea were in the most proximal position above the first cartilage ring; these were not branched as extensively as the controls. More distally, there were no SMGs between the tracheal rings (Fig. 3C,D).

Submucosal Glands Are Absent in *Tabby* (*Eda^{Ta/Y}*) Males and *crinkled* (*Edaradd^{cr}*) Homozygotes

To confirm the observation of Gruneberg that SMGs are absent in *Tabby* mice, we examined histologically the tracheas of four adult

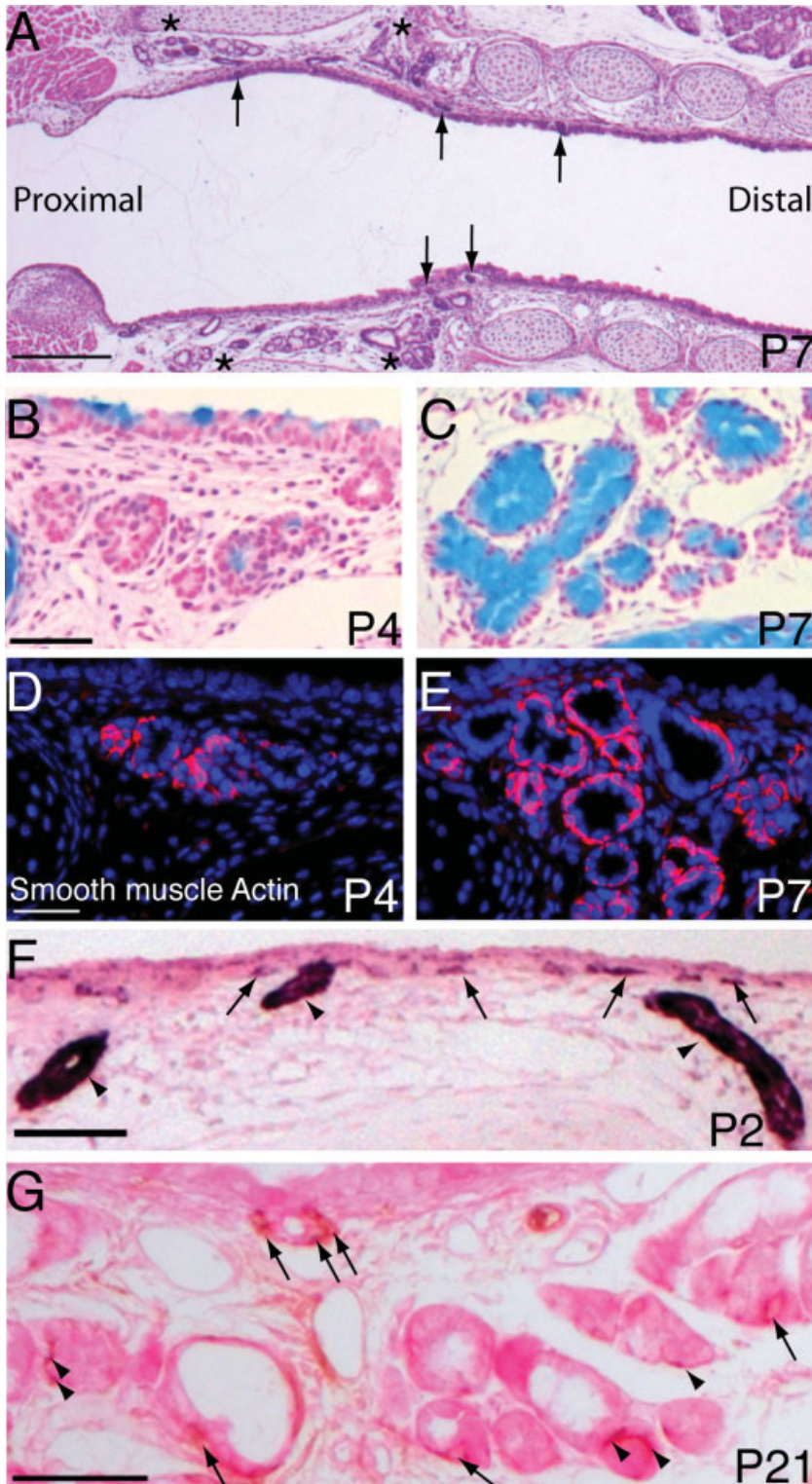


Fig. 1. Normal development of mouse tracheal submucosal glands. A–G: Seven-micrometer paraffin sections of wild-type trachea at postnatal day (P) 2, P4, P7, and P21. **A:** Hematoxylin and eosin staining. Low-magnification overview of the trachea; the proximal (anterior, rostral) end of the trachea is on the left. Asterisks mark well-formed submucosal glands with ducts and branches between the upper tracheal rings. These glands will continue to grow and branch for at least another 2 weeks. Arrows mark early gland buds. **B,C:** Alcian blue staining and nuclear fast red counterstain of P4 (B) and P7 (C) tracheal sections. At P4, the glands are just beginning mucus production; they are apparently fully functional by P7. **D,E:** Anti-smooth muscle actin staining (red) and 4',6-diamidino-2-phenylidole-dihydrochloride (DAPI, blue) counterstain of P4 (D) and P7 (E) tracheal sections. Myoepithelial cells surround the glands at P4 and apparently keep pace with gland development. **F,G:** Anti-cytokeratin 14 staining (dark brown in F, and light brown in G) and eosin counterstain. F: K14 stains the basal cells of the epithelium (arrows) and all the cells of the initial submucosal gland (SMG) buds (arrowheads). G: In mature SMGs, K14 labels basal cells of the ducts and glands (arrows) and myoepithelial cells of the glands (arrowheads). Scale bars = 200 μ m in A,D,E, 500 μ m in B,C,F,G.

Eda^{Ta/Y} males. All samples showed the complete absence of glands (Fig. 4A,B). The mesenchyme beneath the tracheal epithelium where glands should have been present appeared normal and contained blood vessels and calcitonin gene-related peptide (CGRP) positive nerves (data not shown). This analysis clearly established that SMGs are absent in adult animals defective in signaling through the Ectodysplasin pathway. However, it did not distinguish between a failure in the initial formation of the gland primordia and a situation in which glands first form and then regress. This second possibility is consistent with the observation that submandibular salivary glands do form in mice with mutations in the *Eda* pathway, although their subsequent morphogenesis is disrupted (Jaskoll et al., 2003). We therefore analyzed the earliest stages of SMG development in postnatal pups homozygous for a null mutation in *Edaradd*. This gene encodes an essential intracellular adaptor protein that interacts with the death domain of the *Eda*-1 receptor (Headon et al., 2001). Homozygous *crinkled* (*Edaradd*^{cr/cr}) pups at P7 and P14 lacked any sign of newly formed or nascent SMGs (Fig. 4E,F). By contrast, wild-type or heterozygous littermates had both extending and newly formed buds. This result suggests that signaling through the *Eda*-1 receptor (*Edar*) is required for the initial formation of buds, rather than for their subsequent maintenance.

Expression of Components of the *Eda* Signaling Pathway in the Postnatal Trachea and Lungs

The pseudostratified tracheal epithelium of the adult *Eda*^{Ta/Y} mice contained the normal complement of keratin 14-positive basal cells, ciliated cells, and secretory cells. However, individual columnar epithelial cells appeared higher than normal and the epithelium as a whole had a more disorganized appearance (Fig. 4C,D). These changes could be either a direct result of a function for *Eda* signaling in the tracheal epithelium itself, or secondary to the absence of submucosal glands over a prolonged period. The latter explanation is supported by

the observation that the *Edaradd*^{cr} pups do not have an obvious tracheal epithelial phenotype. Nevertheless, to explore possible additional roles for the *Eda* signaling pathway in the trachea and lung, we asked if pathway components continue to be expressed after submucosal gland formation is complete. The genes examined were *Eda*, *Edar*, and *Xedar* and *Tnfrsf19*, which encode related members of the tumor necrosis factor receptor superfamily (Hu et al., 1999; Kojima et al., 2000; Yan et al., 2000). At P49, transcripts from all four genes were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in the upper region of the trachea, where SMGs are present, and also in RNA from more distal tracheal regions and the distal lung (Fig. 5A). Similarly, at P3 and P9, when SMG morphogenesis is occurring, transcripts for all four genes were present throughout the airways (data not shown). In situ hybridization confirmed that *Edar* mRNA is expressed throughout the entire tracheal epithelium at P4 and was not just restricted to the gland-forming regions (Fig. 5B,C).

DISCUSSION

In this study, we document the development of mouse tracheal submucosal glands. We show that they bud from the surface of the tracheal epithelium over the first 3 weeks of postnatal development. In addition, although the earliest-formed glands are apparently functional by P7, they continue to branch and grow until adulthood. Furthermore, we show that the *Eda* signaling pathway is absolutely required for the development of SMGs and that it functions at the earliest stage of SMG formation, namely the initial outgrowth of buds into the mesenchyme. Our analysis raises the possibility that *Eda* and related signaling pathways have additional function(s) in the normal lung and trachea, for example in innate immunity.

The SMGs develop from small epithelial placodes that bud into the mesenchyme in a similar manner to the primordia of the hair follicle, tooth bud, and other ectodermal organs. Like these organs, the SMGs require *Lef1* function for their development, implicating the Wnt intercellular sig-

naling pathway in the formation of the initial buds (Duan et al., 1999). Here, we have shown that the *Eda* signaling pathway is absolutely required for SMG formation, just as it is for the first wave of hair follicles. We therefore asked whether hair and SMG development share any other early signaling events. In the developing hair follicle, *Bmp4* expression is first restricted to the mesenchymal condensation beneath future placodes. Here, it is thought to control follicle spacing by acting as an inhibitor of placode formation in the surrounding regions (Jung et al., 1998; Noramly and Morgan, 1998; Wilson et al., 1999). In contrast, we found that *Bmp4* is expressed throughout the tracheal mesenchyme during the development of the SMGs so that it presumably cannot be functioning to control their spacing. However, *Bmp4* expression does become restricted to mesenchymal cells around the SMGs at late stages in their development and this expression persists into adulthood. Here, by analogy with its presumed role in the intestinal crypts (He et al., 2004), it may function to regulate SMG homeostasis. *Noggin*^{lacZ}, *Patched*^{lacZ}, and *Fgf8*^{LacZ}, which are highly expressed in specific cells of the developing hair follicle and tooth bud, were undetectable in or around the SMGs throughout the P2 to P14 period when morphogenesis is occurring (data not shown).

There is plenty of evidence to support the idea that *Fgf10* plays an important role in the initial budding and subsequent branching morphogenesis of many organs that develop from epithelial placodes (Makarenkova et al., 2000; Ohuchi et al., 2003; Mandler and Neubuser, 2004; Steinberg et al., 2005). In particular, it plays a key role in the distal lung, promoting outgrowth of epithelial buds (Bellusci et al., 1997; Min et al., 1998; Sekine et al., 1999; Weaver et al., 2000). We have found that in *Fgf10* heterozygotes fewer SMGs are formed, and these do not undergo branching morphogenesis to their full extent. This finding supports the notion of general functions for *Fgf10* both in initial budding and branching morphogenesis of these organs. Interestingly, there was a proximal–distal distribution of the severity of the effect on SMGs in the

Fgf10 heterozygotes: the most distal SMGs did not bud at all, the SMGs above the first cartilage element budded and branched but did not complete their branching morphogenesis, and the morphogenesis of the more proximal nasal glands was apparently normal (data not shown). This finding suggests that either there is a proximal–distal difference in the requirement of the glands for *Fgf10* or, more likely, that other Fgfs are compensating in the more proximal regions. *Fgf10* is typically transcribed in the mesenchyme adjacent to budding organs, these include the lacrimal gland, feather placodes, and distal lung buds (Bellusci et al., 1997; Makarenkova et al., 2000; Mandler and Neubuser, 2004).

Both Wnt and Eda signaling are required for the formation of the first wave of hair and feather follicles and in tooth development, but the precise connection between these two signaling pathways, and other intercellular signaling events, is still unclear. In vitro experiments have suggested that Eda is directly regulated by Wnt signaling in the tooth bud (Pengue et al., 1999; Laurikkala et al., 2001; Durmowicz et al., 2002).

More recently, it has been suggested that Eda signaling is a competence factor for placode formation in the early stages of ectodermal organ development (Mustonen et al., 2004; Houghton et al., 2005). Both of these hypotheses are consistent with our data for an early role of Eda signaling in SMG development. Interestingly, overexpression of Eda ligand itself in adult mice cannot rescue the *Tabby* phenotype but can cause sebaceous gland hyperplasia in wild-type animals (Cui et al., 2003; Gaide and Schneider, 2003; Mustonen et al., 2003). Therefore, it is tempting to speculate that an increase in levels of Eda signaling plays a role in the SMG hyperplasia observed in severe asthma and other respiratory diseases.

We have shown that *Eda*, *Edar*, and two related receptors, *Xedar* and *Tnfrsf19* are expressed in the lower trachea and lung, both in the early postnatal period and in the adult. In addition, *Traf 4*, which encodes an adaptor protein for some TNF receptor family members, is expressed in airway epithelial cells, and homozygous mutants have a constriction of the upper trachea, although SMGs

have not been studied (Krajewska et al., 1998; Shiels et al., 2000; Regnier et al., 2002). These data suggest that signaling through the TNF pathway has important roles not only in the development of the SMGs but also in the normal function of the rest of the lung. These pathways all converge on the Rel/Nf-kB complex, which is translocated to the nucleus to regulate gene transcription. Recent studies have shown that bronchial epithelial cells express nuclear RelA in response to allergen or lipopolysaccharide-induced inflammation (Poynter et al., 2002, 2003, 2004). This is evidence that Nf-kB can act directly within airway epithelial cells but the downstream targets are not yet known. Although the defects that we observed in the morphology of the airway epithelial cells of *Eda* mutant mice are probably a long-term consequence of life without submucosal glands, it is still possible that the Eda signaling pathway mediates some responses to injury or inflammation within the epithelium. *Tabby* and *crinkled* mutant mice represent useful models with which to explore these possibilities.

Fig. 2. *Bmp4* expression around the tracheal submucosal glands (SMGs). A–C: Seven-micrometer paraffin sections of *Bmp4*^{LacZ/+} trachea at postnatal day (P) 2, P8, and P28 stained with X-gal (blue) to show expression and counterstained with eosin. **A:** At P2, *Bmp4* is expressed around existing gland buds (arrows) and also throughout the tracheal mesenchyme (not shown) and around the cartilage. **B:** At P8, *Bmp4* expression is still widespread in the tracheal mesenchyme but is also beginning to appear around the SMGs themselves. **C:** By P28, *Bmp4* is restricted to a subset of mesenchymal cells around the SMGs. It remains on in these cells throughout later life. It is also expressed in a few mesenchymal cells directly underneath the tracheal epithelium (inset). **D:** Seven-micrometer paraffin section of P28 wild-type trachea, stained with X-gal as in A–C and counterstained with eosin. There is no X-gal reactivity. Scale bars = 500 μ m in A,B, 200 μ m in C,D.

Fig. 3. Tracheal submucosal glands are hypoplastic in *Fgf10* heterozygotes. A–D: Seven-micrometer paraffin sections of *Fgf10*^{+/+} and *Fgf10*^{+/-} postnatal day (P) 20 trachea; Alcian blue and Nuclear fast red staining. **A,B:** *Fgf10*^{+/+}. Note the presence of submucosal glands (SMGs) down to the third tracheal ring. The SMGs above the first tracheal ring are the most branched. **C,D:** *Fgf10*^{+/-}. The SMGs are not present below the first tracheal ring (arrows mark the most distal SMG in each panel). The remaining SMGs have not branched as extensively as those in A and B. An arrowhead labels a gland duct in D, it is associated with a small gland that is not visible in this section. The extent of SMG branching visible in each section through a trachea is highly dependent on the precise angle at which the trachea is embedded; hence, the section of each trachea that was imaged was the one in which most SMG branches were visible. The tracheal rings are numbered in a proximal–distal sequence. T labels the thyroid gland. Scale bars = 200 μ m in A–D.

Fig. 4. Absence of tracheal submucosal glands in mutants of the Ectodysplasin signaling pathway. **A,B:** Alcian blue staining of wild-type (*Eda*^{+/+}) (A) and *Tabby* (*Eda*^{Ta/Y}) mutant (B) upper trachea at postnatal day (P) 6 weeks. B: Arrows mark where the submucosal glands (SMGs) should be. **C,D:** Anti-cytokeratin 14 staining of *Eda*^{+/+} (C) and *Eda*^{Ta/Y} (D) trachea at P6 weeks. Basal (K14-positive) cells are present in both genotypes, but note the increased height of the epithelium in D. **E,F:** Hematoxylin and eosin staining of wild-type (*Edar*^{+/+}) (E) and *crinkled* (*Edar*^{cr/cr}) (F) trachea at P7. Fully formed and budding (arrow) SMGs are present in the wild-type but not the mutant. **A,C:** P6 weeks wild-type *Eda*^{+/+} 7- μ m paraffin sections. **B,D:** P6 weeks *Eda*^{Ta/Y} 7- μ m paraffin sections. **E:** P7 wild-type *Edar*^{+/+} 7- μ m paraffin section. **F:** P7 *Edar*^{cr/cr} 7- μ m paraffin section. Scale bars = 200 μ m in A,B, 20 μ m in C,D, 500 μ m in E,F.

Fig. 5. Expression of Ectodysplasin signaling pathway components in the adult and developing trachea. **A:** Reverse transcriptase-polymerase chain reaction using *Eda*-, *Edar*-, *Xedar*-, and *Tnfrsf19*-specific primers from postnatal day (P) 49 RNA samples. Templates: 1. P49 upper trachea (proximal to the third tracheal ring) cDNA, 3. P49 lower trachea (distal to the third tracheal ring) cDNA, 5. P49 distal lung cDNA, 7. P49 esophagus cDNA. 2, 4, 6, and 8 are corresponding RNA controls. **B,C:** Radioactive in situ hybridization of upper trachea using antisense (B) and sense (C) *Edar* probes (black grains) counterstained with hematoxylin (purple). Wild-type P4 tracheal sections. The *Edar* RNA is present throughout the entire tracheal epithelium. Scale bars = 200 μ m in B,C.

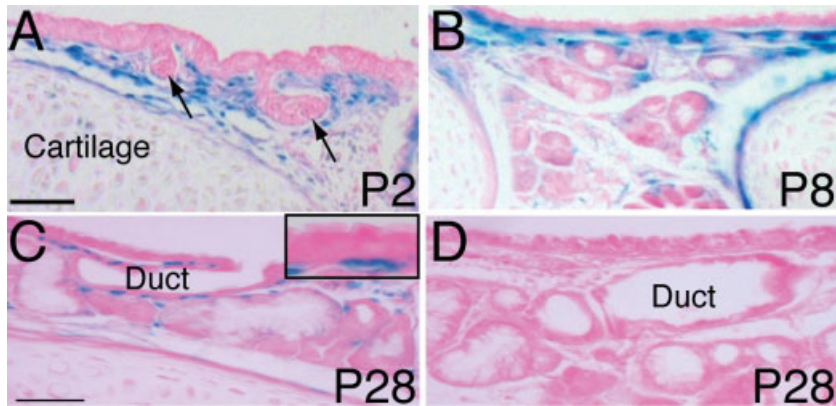


Fig. 2.

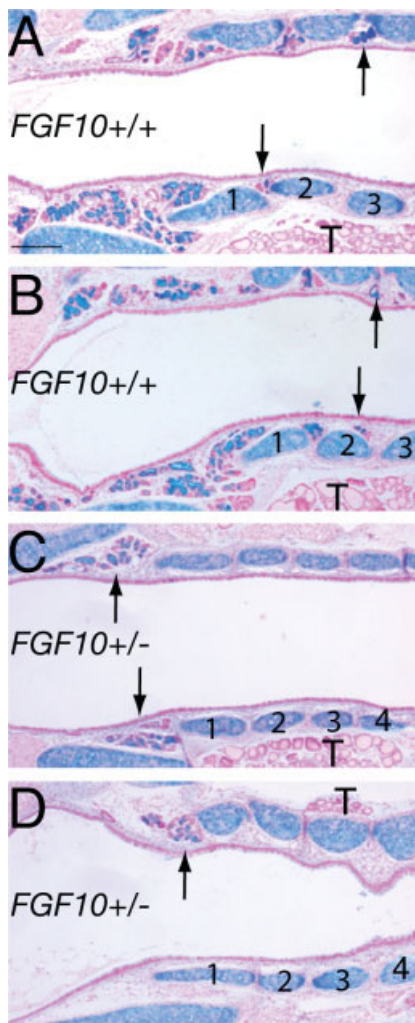


Fig. 3.

EXPERIMENTAL PROCEDURES

Animals

Wild-type animals were ICR outbred mice (Harlan Sprague-Dawley, India-

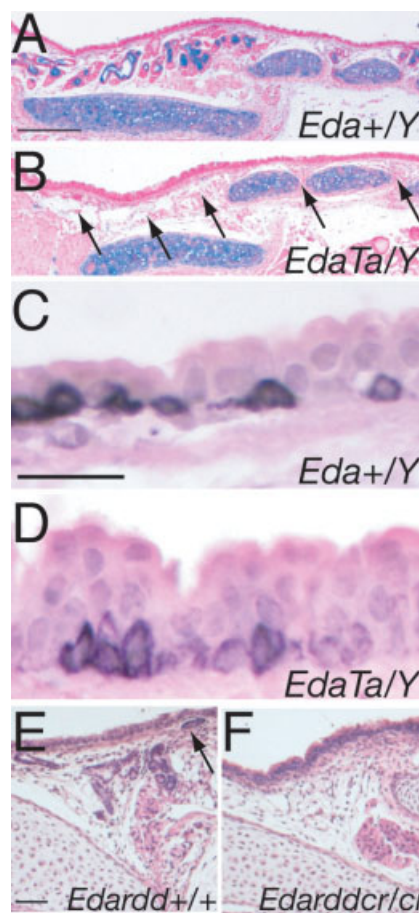


Fig. 4.

napolis, IN). *Eda^{Ta/Y}* adult males (four males from two litters with wild-type siblings) and *Edaradd^{cr/+}* breeding pairs were purchased from the Jackson Laboratory. *BMP4^{LacZ}* and *Fgf10^{+/-}* heterozygotes as described, were maintained on an outbred genetic background (Lawson et al., 1999; Sekine et al., 1999).

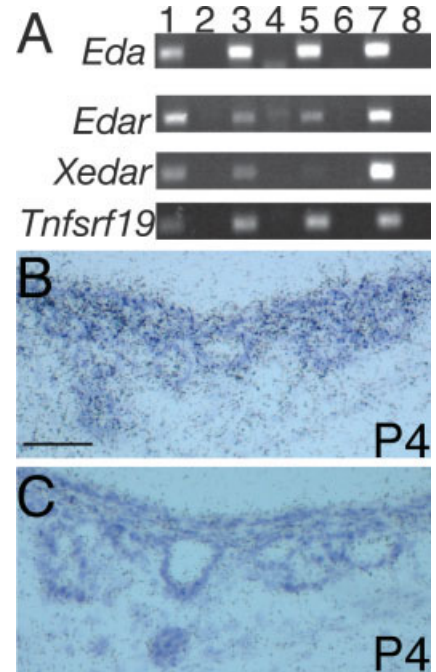


Fig. 5.

In Situ Hybridization

For section in situ hybridization, wild-type trachea were fixed in 4% paraformaldehyde/phosphate buffered saline, dehydrated, and embedded in paraffin. Seven-micrometer sections were used for in situ hybridization with ³⁵S-labeled sense and antisense probes. These were prepared from a 1.4-kb fragment of *Edar* (Laurikkala et al., 2001) using the Boehringer–Mannheim RNA labeling kit. Slides were exposed to emulsion for up to 10 days and counterstained with hematoxylin.

β -Galactosidase Expression

Bmp4^{LacZ} heterozygotes were mated with ICR mice. Trachea were excised from the pups from P0 to P28 fixed in 4% paraformaldehyde/phosphate buffered saline (PBS), permeabilized, and stained for LacZ from overnight to 2 days. They were subsequently embedded in paraffin, sectioned at 7 μ m, and counterstained with eosin.

Immunohistochemistry and Alcian Blue Histology

Seven-micrometer sections of paraffin-embedded tissue were dewaxed and rehydrated before antibody staining; immunohistochemical reactions

were carried out in parallel with control reactions lacking primary antibodies. Primary antibodies used were mouse monoclonal anti- α -smooth muscle actin (Clone 1A4, Sigma, St. Louis, MO) 1:200 dilution after protease antigen retrieval, mouse monoclonal K14 antibody (Clone LL002, Neomarkers, Fremont, CA) 1:200 dilution after antigen retrieval by microwave treatment in citrate buffer, and rabbit anti-CGRP (Peninsula Laboratories, Inc., San Carlos, CA) 1:1,000 dilution. Primary antibodies were incubated at 4°C overnight. Secondary antibodies were goat anti-mouse Cy3 from Jackson ImmunoResearch (West Grove, PA) and biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA). For the biotinylated antibody, the signal was amplified using the Vectastain Elite ABC kit and visualized with diaminobenzidine (DAB) substrate (Vector Laboratories). Sections were counterstained with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) or eosin.

Alcian blue staining for acid mucins was performed on 7- μ m sections of paraffin-embedded tissue, which were dewaxed and rehydrated to water, incubated in Alcian blue pH 2.5 (1% Alcian blue in 3% acetic acid) for 5 min, washed in water, and counterstained with 0.1% Nuclear fast red for 5 min and washed in water. Sections were then dehydrated, cleared, and mounted in Permount (Fisher, Pittsburgh, PA).

BrdU Incorporation

To detect cell proliferation, BrdU (Amersham Biosciences, Piscataway, NJ) was diluted in PBS and injected intraperitoneally into pups at a dose of 10 μ l per gram body weight. BrdU was detected using mouse monoclonal anti BrdU (Sigma-Aldrich, St. Louis, MO) with heating in dilute HCl, microwave treatment in citrate buffer and protease digestion for antigen retrieval. The Mouse on Mouse Kit and DAB (Vector Laboratories) were used to detect the antigen-antibody complexes.

RT-PCR

Total RNA was extracted from the upper and lower trachea and the left lobe

of the lung at P3, P9, and P49 using the Rneasy kit (QIAGEN, Inc., Valencia, CA). The cDNA was synthesized from 1 μ g of total RNA using the Superscript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA). Thirty cycles of amplification were performed using the following primer pairs: β -actin, 5'-GTCTGACACAGGCATTGTGATGG-3' and 5'-GCAATGCCTGGGTACATGGTGG-3'; EDA, 5'-GAGAAGCAAAAGTGGTGAAG-3' and 5'-GTTTCATAGTGATGCGAGACC-3'; Edar, 5'-TGTGTATGCCAACGTGTGTG-3' and 5'-TCCTTCATTTGCCTAGGTG-3'; Xedar, 5'-CCCTCTACTGGACCTGAAAC-3' and 5'-CAGGCAAACCTCCACCTCATT-3'; TNFSRF19, 5'-TGTCACCACCCAGAGGATTC-3' and 5'-CGTCCTTGTGCTGTGAAGAG-3'.

ACKNOWLEDGMENTS

The authors thank Irma Thesleff for Eda probes; Eric Meyers, Matt Godderis, and Roger Ilgan for providing *Fgf8^{LacZ}*, *Ptc^{LacZ}*, and *Fgf10* heterozygous mice; and Scott Randell for generous discussion and advice.

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