Ectodysplasin Receptor-Mediated Signaling Is Essential for Embryonic Submandibular Salivary Gland Development

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ABSTRACT

Hypohidrotic (anhidrotic) ectodermal dysplasia (HED), the most common of the ~150 described ectodermal dysplasias, is a disorder characterized by abnormal hair, teeth, sweat glands, and salivary glands. Mutations in the *EDA* (*ectodysplasin-A*) and *EDAR* (*ectodysplasin-A receptor*) genes are responsible for X-linked and autosomal HED, respectively. Abnormal phenotypes similar to HED are seen in *Tabby* (Eda^{Ta}) and *downless* ($Edar^{dl}$) mutant mice. Although recent studies have focused on the role of Eda/Edar signaling during hair and tooth development, very little is known about its role during embryonic submandibular salivary gland (SMG) development. To this end, we analyzed the SMG phenotypes in *Tabby* (*Ta*) and *downless* (*dl*) mutant mice and determined that *Ta* SMGs are hypoplastic, whereas *dl* SMGs are severely dysplastic. The absence of SMG ducts and acini in *dl* SMGs suggests that Eda/Edar signaling is essential for lumina formation and glandular histodifferentiation. Our localization of Eda and Edar proteins at sites of lumen and acini formation supports this conclusion. Moreover, the presence of SMGs in both *Ta* and *dl* mutant mice, as well as the absence of immunodetectable Eda and Edar protein in Initial Bud and Early Pseudoglandular stage SMGs, indicate that Eda/Edar-mediated signaling is important for branching morphogenesis and histodifferentiation, but not for initial gland formation. To initially delineate the morphoregulatory role of Eda/Edar-mediated signaling during embryonic SMG development, we cultured embryonic day 14 SMGs with enhanced or abrogated Eda/Edar signaling. Eda supplementation induced a significant increase in SMG branching, and enhanced activation of NF-κB. Abrogating Eda/Edar signaling by adding the soluble form of Edar to bind endogenous ligand in embryonic SMGs results in a significant dose-dependent decrease in branching morphogenesis. Taken together, our results suggest that the Eda/Edar/NF-κB pathway exerts its effect on SMG epithelial cell proliferation, lumina formation, and histodifferentiation. Anat Rec Part A 271A:322–331, 2003.

Key words: ectodysplasin; ectodysplasin receptor; hypohidrotic ectodermal dysplasia; embryonic salivary gland; NF-κB activation; branching morphogenesis; Tabby; downless

Ectodysplasin-A (Eda), a protein required for epithelial morphogenesis, is encoded by the X-linked *EDA* gene (Srivastava et al., 1997). Mutations in *EDA* or its mouse homologue *Eda* (formerly *Tabby*) give rise to the syndrome hypohidrotic ectodermal dysplasia (HED) (Kere et al., 1996; Srivastava et al., 1997; Monreal et al., 1998), which is characterized by absent or hypoplastic teeth, hair, sweat glands, and salivary glands (Gruneberg, 1965, 1971; Blecher et al., 1983; Clarke et al., 1987; Nordgarden et al., 1998, 2001). A similar phenotype is seen in *downless* (*dl*) mutant mice due to mutations in the *Edar* (formerly *downless*) gene, the mouse homologue of the human *EDAR* (Eda receptor) gene (Headon and Overbeek, 1999). Significantly, mutations in *EDAR* have been found in several families with autosomal forms of HED and in *dl* mice (Monreal et al., 1998). All affected structures (e.g., teeth, sweat glands, hair follicles, and salivary glands) are derived from interactions between the epithelium and mes-

Grant sponsor NIH; Grant number: DE 11942.

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Received 10 October 2002; Accepted 11 November 2002

DOI 10.1002/ar.a.10045

Published online 10 March 2003 in Wiley InterScience (www.interscience.wiley.com).

enchyme (Gruneberg, 1965; Wessells, 1997). Although recent studies have focused on the role of Eda and Edar during hair and tooth development (Mikkola et al., 1999; Pispa et al., 1999; Tucker et al., 2000; Yan et al., 2000; Laurikkala et al., 2001, 2002), very little is known about the role of the Eda/Edar signal transduction pathway during embryonic salivary gland development.

Embryonic SMG Development

The neonatal SMG is comprised of a network of large and small ducts that terminate in lumen-containing, presumptive acini that express the embryonic mucin (Jaskoll et al., 1998; Melnick et al., 2001c). Progressive prenatal morphogenesis begins as a solid outgrowth from the oral epithelium around embryonic day 11.5 (E11.5). To arrive at its newborn anatomy, the SMG bud undergoes cell proliferation and apoptosis, which are mediated by the appearance of growth factors, cytokines, and transcription factors at specific times and places (Jaskoll and Melnick, 1999; Melnick and Jaskoll, 2000). Mechanistic and gene-targeting experiments have demonstrated the importance of several signaling pathways, including TGF-α/EGF/EGFR, TNF/TNFR, FGF/FGFR2, IL-6/IL-6R, and TGF-β/TGF-βRII (Hardman et al., 1994; Gresik et al., 1995; Kashimata and Gresik, 1997; Jaskoll et al., 1998, 2002; De Moerlooze et al., 2000; Revest et al., 2001; Melnick et al., 2001a, b, d; Jaskoll and Melnick, 2002). However, it is clear that other, as yet unidentified signal transduction pathways also play critical morphoregulatory roles during SMG development.

Eda/Edar-Mediated Signaling

Eda and Edar proteins are a novel TNF-like transmembrane ligand and receptor, respectively (Ferguson et al., 1997; Ezer et al., 1999; Headon and Overbeek, 1999). Eda is a glycosylated, oligomeric type II membrane protein (Ezer et al., 1997; Ferguson et al., 1997; Srivastava et al., 1997), with three collagenous repeat domains and a TNF homology domain. Both the human *EDA* gene and its mouse homolog, *Eda,* undergo extensive alternative splicing to produce several isoforms, including Eda-A1 and Eda-A2 (Srivastava et al., 1997; Bayes et al., 1998; Monreal et al., 1998; Yan et al., 2000). Eda-A1 is shed from the cell membrane as a soluble ligand and binds as a trimer to its trimerized Eda receptor (Edar) (Chen et al., 1997; Elomaa et al., 2001). The primary biologically active isoform is Eda-A1, which binds specifically to Edar and not to other known TNF receptors (Yan et al., 2000; Tucker et al., 2000; Kumar et al., 2001). A second receptor, Xedar, has been mapped to the X chromosome and shown to be the receptor for Eda-A2 (Yan et al., 2000); however, it has not been shown to be related to human HED and mouse *downless* phenotypes. (In the present work, Eda is used to designate the biologically active ligand, Eda-A1.)

Like most genes in the TNF-receptor superfamily, *Edar* encodes a type I transmembrane glycoprotein with an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic region that activates cell functions (Darnay and Aggarwal, 1997; Wallach et al., 1999). Although all TNF-related apoptosis-inducing receptors share a C-terminal domain called the "death domain" (Yuan, 1997; Ashkenazi and Dixit, 1998), Edar possesses a "death domain" that does not interact with TRADD or FADD, the death domain-containing adaptor proteins associated with TNF- or FAS-mediated death (Kumar et al., 2001). Rather, the Edar-associated death domain (Edardd) adaptor is encoded by the mouse *crinkled* locus. Edardd interacts with Edar's death domain to link the receptor to downstream signaling pathways (Headon et al., 2001; Yan et al., 2000). Specifically, Eda/Edar/Edardd signaling has been shown to activate NF-κB (Yan et al., 2000; Kumar et al., 2001; Doffinger et al., 2001; Headon et al., 2001). Overexpression of Eda, Edar, or Edardd activates NF-κB, whereas mutations in Eda, Edar, or Edardd reduce or abolishe NF-κB's activation (Headon et al., 2001; Koppinen et al., 2001; Yan et al., 2000). In addition, Edar signaling also weakly induces the JNK- and caspase-independent death pathways (Kumar et al., 2001). Unlike TNF- or FAS-mediated cell death, Eda/Edar-mediated death is neither caspase-dependent nor inhibited by NF-κB (Kumar et al., 2001).

Our previous studies demonstrated that TNF/TNFR signaling plays a important role in balancing mitogenesis and apoptosis during embryonic SMG development (Melnick et al., 2001a). Moreover, since the NF-κB cascade is the primary downstream pathway induced by TNF ligand/receptor binding, we also investigated the role of NF-κB during embryonic SMG morphogenesis (Melnick et al., 2001b). Interruption of NF-κB activation in vitro resulted in a significant increase in apoptosis and a highly significant decrease in cell proliferation, as well as the altered expression of genes and proteins involved in translational control, cell cycle progression, apoptosis, and signal transduction. Based on these results, we concluded that NF-κB signaling plays a critical role during embryonic SMG development. Given that: 1) members of the TNF/TNFR superfamily have been shown to play an essential role during embryonic SMG development (Melnick et al., 2001a); 2) NF-κB activation plays an important morphoregulatory role for embryonic SMG epithelial cell proliferation and survival (Melnick et al., 2001b); 3) Eda/Edar signaling activates NF-κB (Yan et al., 2000; Kumar et al., 2001; Koppinen et al., 2001); and 4) mice with suppressed NF-κB/Rel exhibit defects in hair, exocrine glands, and tooth development identical to those seen in Eda (*Tabby*) and Edar (*downless*) mutant mice (Schmidt-Ullrich et al., 2001), it was reasonable to postulate that Eda/Edar plays an important role during embryonic SMG development, likely through the NF-κB cascade.

In this study, we investigated the relationship between Eda/Edar signaling and embryonic SMG morphogenesis and histodifferentiation. Our results indicate that the Eda/Edar/NF-κB pathway likely plays an important morphoregulatory role during embryonic SMG development.

MATERIALS AND METHODS

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Jaskoll and Melnick, 1999); plug day = day 0 of gestation. All studies involving these animals were conducted in a humane manner. The Animal Review Board and Vivaria Advisory Committee reviewed all applications to ensure ethical and humane treatment of the animals. These bodies follow the *NIH Guide for the Care and Use of Laboratory Animals* (revised 1985) of the Institute of Laboratory Resources, National Research Council guidelines, all applicable government regulations, and the USC policies governing the care and use of laboratory animals. The animals are killed according to these

established guidelines. All mice are housed in an approved vivarium. The vivarium facility is temperature-controlled, with an average daily temperature of 24°C. Alternate 12-hr periods of light and darkness are maintained daily. Our colony has been kept virtually disease-free by regular periodic checks for bacterial and viral diseases.

Pregnant females were euthanized by cervical dislocation on days 13–18 of gestation (E13–18); newborn mice were euthanized on postnatal day 1. Embryos were dissected in cold phosphate-buffered saline (PBS) and staged according to Theiler (1989). E13 heads and E14-newborn SMGs were dissected, collected, and processed for histology.

Immunolocalization of Eda and Edar Proteins

To determine the cell-specific distribution of Eda and Edar in SMG development, we evaluated E13-newborn SMGs by immunohistochemistry essentially as previously described (Jaskoll and Melnick, 1999). Briefly, embryonic heads (E13) or SMGs (E14-newborn) were fixed in Carnoy's fixative, embedded in low-melting-point paraplast, and stored at 4°C. Tissues were serially sectioned at 5μm, placed on cleaned, gelatin-coated slides at 37°C for 3 hr, and immediately immunostained using affinity-purified, peptide-specific rabbit polyclonal antibodies to Eda and Edar. Controls consisted of sections incubated with preimmune serum or no primary antibodies. Peptide-specific polyclonal antibodies were generated and their specificity was characterized in our laboratory. A rabbit polyclonal antibody to Eda was raised against a 21 aa peptide [(C)K-AYSEESRRVRRNKRSKS-COOH] representing amino acids #144~162 of the deduced mouse Eda amino acid sequence (Srivastava et al., 1997); a rabbit polyclonal antibody to Edar was raised against an 18 aa peptide [RPGEEPYMSCGYGTKDDDY-COOH] representing amino acids #51~69 of the deduced mouse Edar amino acid sequence (Headon and Overbeek, 1999). Each antibody was purified from the immune sera by affinity chromatography on a peptide column, and its specificity was determined by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

Characterization of *Tabby* and *downless* Mutant Mice

We purchased 9-week-old male *Tabby* (Eda^{Ta}/Y) mice and their wild-type littermates (+/Y) from Jackson Laboratories (Bar Harbor, ME). Since Eda^{Ta} is X-linked, the *Ta* phenotype is seen in both hemizygous males and homozygous females. We purchased 7–9-week-old female *downless* ($Edar^{dl\text{-}J}/Edar^{dl\text{-}J}$) mice and their wild-type littermates from Jackson Laboratories. The SMGs were dissected and processed for histological or protein analyses as previously described (Melnick et al., 2001a). For morphological analysis, the SMGs were sectioned and stained with hematoxylin and eosin. For analysis of histodifferentiation, mucin protein immunolocalization was conducted as described in Jaskoll et al. (1998); controls consisted of sections incubated with preimmune serum. For protein analyses, the total protein level per gland was determined by the Bradford method, and the mucin protein level per mg/protein was determined by ELISA essentially as described in Harlow and Lane (1988).

Culture System

E14 SMG (Late Pseudoglandular stage) primordia were cultured using a modified Trowell method as previously described (Melnick et al., 2001a). The medium consisted of BGJb (Life Technologies, Rockville, MD) supplemented with 0.5 mg ascorbic acid/ml and 50 U penicillin/streptomycin (Life Technologies), pH 7.2. Replicate cultures were changed every other day. For supplementation studies, paired E14 SMG primordia were cultured in the presence or absence of exogenous Eda ligand (0.5 μg/ml Flag-Eda-A1) for 3 days, and the Spooner branch ratio (terminal bud number after 3 days/initial bud number) was determined for each explant. Abrogation experiments consisted of adding exogenous soluble Edar-Fc chimera to the culture medium to competitively bind endogenous Eda and any other Edar ligands. This receptor/ligand binding methodology was previously used to interrupt tooth development in vitro (Tucker et al., 2000); controls consisted of sections incubated with BGJb + BSA alone. Paired E14 SMG primordia were cultured with 100 or 150 ng/ml hEdar-Fc supplementation (Genentech, South San Francisco, CA) or control medium for 3 days, and the Spooner branch ratios were determined as described above. The data were then arcsin-transformed and the mean ratios compared by paired *t*-test (Sokal and Rohlf, 1981). It is important to note that since a notable difference in SMG branch number is seen among littermates, we compared the number of terminal buds in the right and left glands (treated and control) from each embryo by paired *t*-test for all embryos studied. In each experiment, a minimum of five explants were evaluated.

To determine whether enhanced Eda/Edar signaling induced NF-κB activation, paired E14 SMGs were cultured for 6 days in the presence or absence of 0.5 μg/ml Flag-Eda-A1 supplementation. Since NF-κB was not immunodetected until the canalicular stage, and the rate of branching morphogenesis and histodifferentiation is substantially slower in vitro than in utero, we lengthened the E14 (pseudoglandular stage) SMG culture period from 3 to 6 days to achieve the canalicular stage. Explants were collected and evaluated by histological and immunochemical assays as described above. In this set of experiments, we used a polyclonal goat anti-NF-κB p65/RelA antibody (C-20)(Santa Cruz Biotechnology, Santa Cruz, CA), which has been shown to cross-react with mouse p65; it is not cross-reactive with RelB p68 or c-Rel p75. Controls consisted of sections incubated with PBS alone.

RESULTS

Eda and Edar Localization in SMG Epithelia

To begin to understand the role of Eda/Edar signaling during embryonic SMG development, we analyzed the cell-specific distribution of Eda and Edar proteins in Pseudoglandular (~E14), Canalicular (~E15-E16), Terminal Bud (~E17-E18), and neonatal SMGs. Eda and Edar proteins are not immunodetected until the Late Pseudoglandular/Early Canalicular stage. With the onset of lumen formation in the early canalicular stage, Eda protein is immunolocalized to epithelial surfaces at sites of lumen formation, and on a few terminal bud cells at sites of future cell death (Fig. 1A). By contrast, Edar protein is distributed on membranes of ductal and terminal bud epithelia, with more intense immunostaining seen on lumen-bounding cells (Fig. 1B). With the appearance of distinct ductal and terminal bud lumina in the Late Canalicular and Terminal Bud stages, Eda and Edar pro-

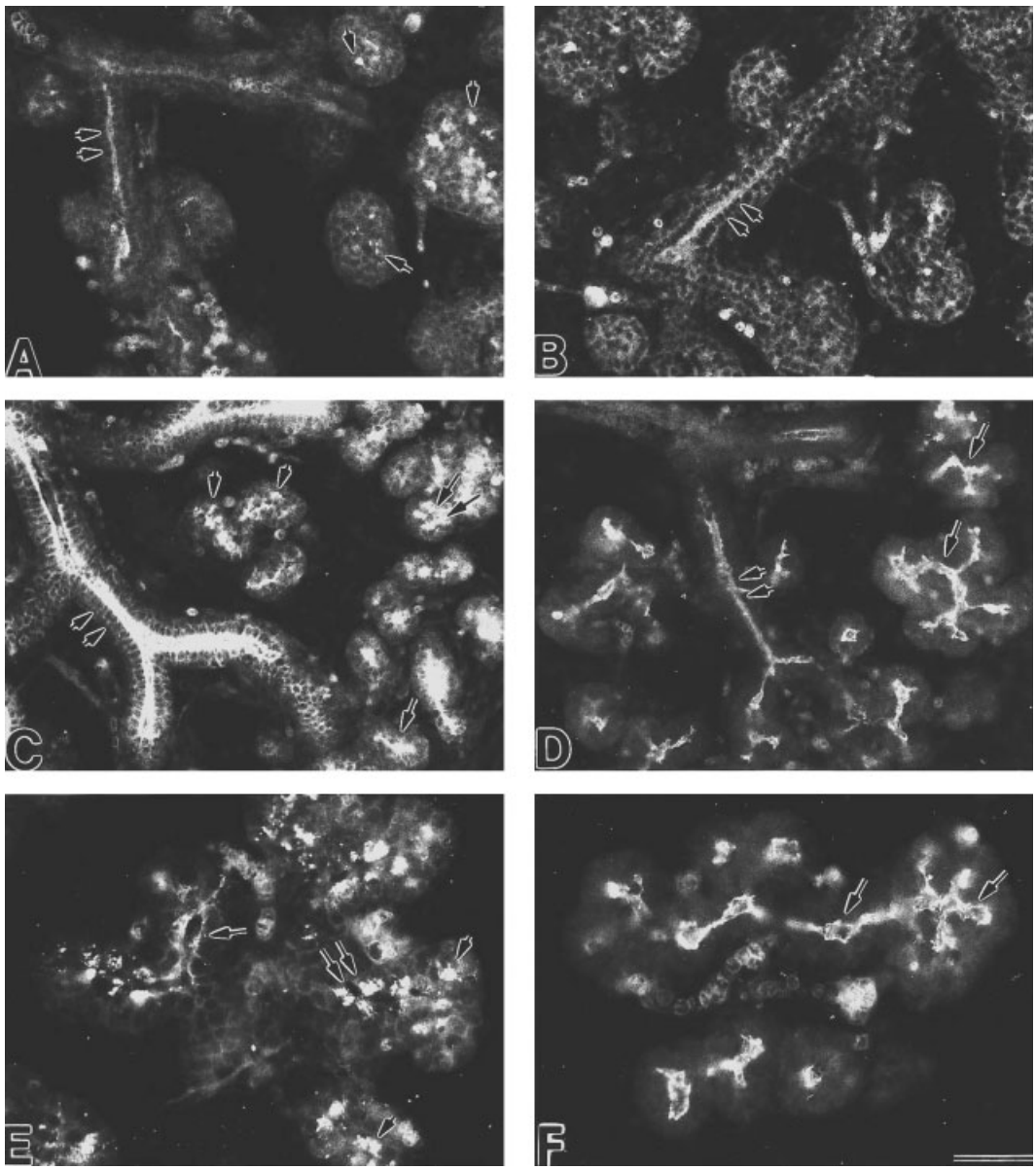

Fig. 1. Epithelial distribution of Eda and Edar in embryonic SMGs. **A** and **B:** In the Early Canalicular stage, (A) Eda is immunodetected on epithelial surfaces at sites of ductal lumen formation (double arrowheads) and on terminal bud cells destined to die (arrowhead); (B) Edar is seen on the membranes of ductal (double arrowheads) and terminal bud epithelia, with more intense immunostaining seen at sites of lumen formation. **C–F:** In the Terminal Bud stage, (C and E) Eda and (D and F) Edar are seen on the apical surface of epithelial cells bounding ductal (double arrowheads) and terminal bud (arrow) lumina. Eda also appears to be localized within cells surrounding newly-forming terminal bud lumina (double arrows) and within terminal bud epithelial cells destined to die (arrowhead). Bar = (A–D) 50 μm and (E–F) 25 μm.

teins are seen on the apical surfaces of lumen-bounding epithelia (Fig. 1C–F). Eda also appears to be within cells surrounding terminal bud lumina (Fig. 1C) and individual terminal bud cells, which according to our previous studies (Jaskoll and Melnick, 1999) are destined to die (Fig. 1E). This distribution pattern persists in the newborn SMG (data not shown).

Abnormal SMG Phenotypes in *Tabby* and *downless* Mutant Mice

The *Ta* (Eda^{Ta}) phenotype is caused by a truncated, loss-of-function Eda ligand (Srivastava et al., 1997; Ferguson et al., 1997), whereas the *dl* ($Edar^{dl}$) phenotype is due the loss-of-function Eda receptor (Headon and Overbeek, 1999). We evaluated and compared *Ta* and *dl* ($dl^{Jackson}$) SMG morphology and mucin protein expression in postnatal mice, since acinar histodifferentiation occurs postnatally (Srinivasan and Chang, 1979). Both *Ta* and *dl* mice exhibit abnormal SMG phenotypes, with marked differences between mutant strains (Figs. 2–6). The *Ta* SMG is substantially smaller than the wild-type control gland and is characterized by decreased granular convoluted ducts and acini (Fig. 2). This observation is similar to that reported by Blecher et al. (1983). Although acinar-

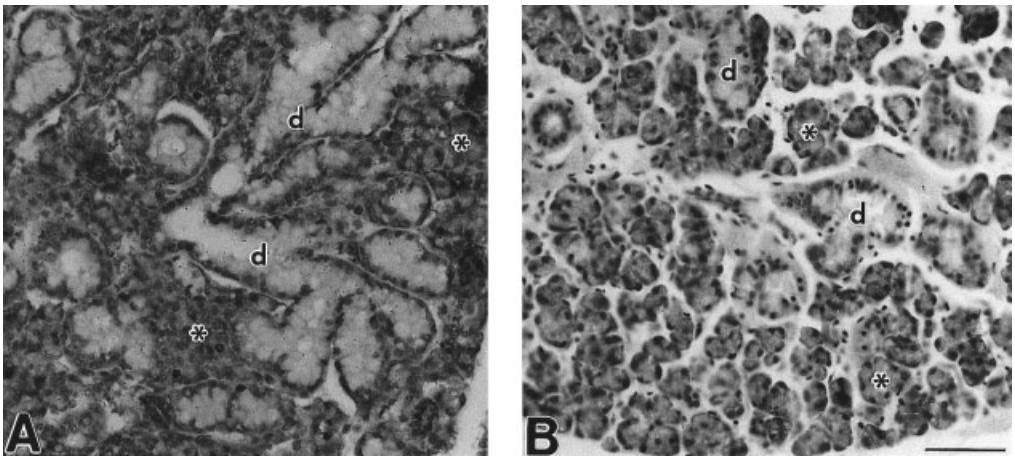

Fig. 2. *Tabby* SMGs are hypoplastic. Compared to (**A**) wild-type controls, (**B**) *Ta* (*Eda*^{Ta}/*Y*) hemizygous SMGs exhibit a decrease in duct (d) size and number and smaller acini (*). Note that since the *Ta* SMGs are smaller than the wild-type control glands, the region shown in B represents a greater proportion of the total *Ta* gland than A represents for the wild-type gland. Bar = 50 μm.

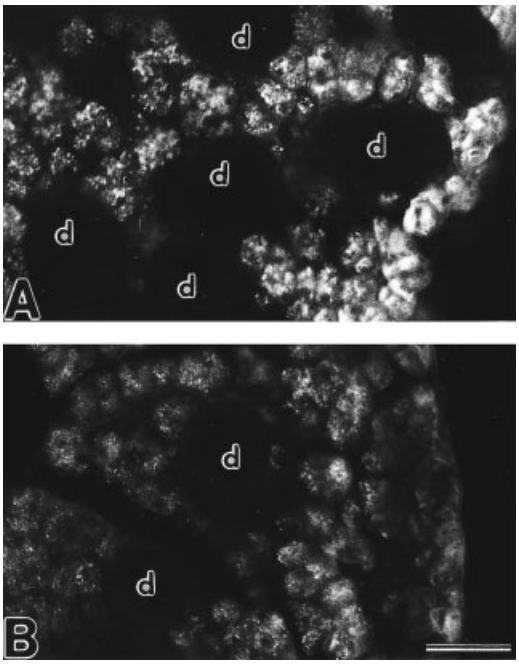

Fig. 3. *Ta* SMGs exhibit a notable decrease in immunodetectable mucin protein. Mucin protein immunolocalization was compared in (**A**) wild-type and (**B**) *Ta* (*Eda*^{Ta}/*Y*) hemizygous SMGs. A marked decrease in immunodetectable mucin protein in *Ta* acinar cells (*) is seen. D, duct. Bar = 50 μm.

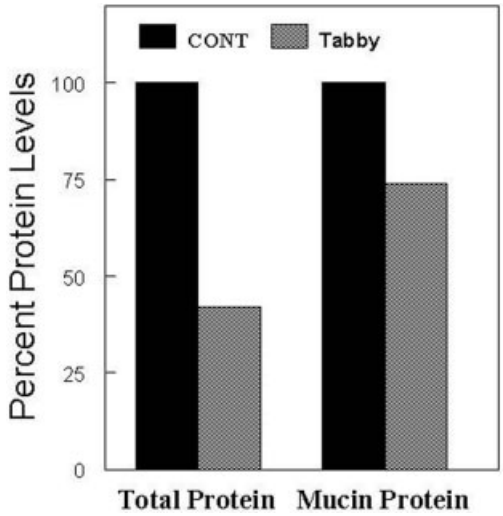

Fig. 4. *Ta* SMGs exhibit a significant decrease in total protein and mucin protein levels compared to control (CONT) littermates. SMGs were collected from *Ta* and their wild-type littermates, the total protein level per gland was determined, and the means were calculated. The mucin protein level per gland was determined by ELISA, and the means were calculated. Data were analyzed by *t*-test. Two or three glands per genotype were analyzed.

specific mucin protein is localized in both control and *Ta* acinar cells, we see a notable decrease in the relative level of immunolocalized mucin in the hypoplastic *Ta* SMGs compared to the wild-type controls (compare Fig. 3B with 3A). Quantitation of total and mucin protein levels in *Ta* and control SMGs reveals a significant 58% decrease ($t = 10.91$, $P < 0.001$) in the total protein level, and a significant 26% decrease ($t = 5.08$, $P < 0.02$) in the mucin protein level in *Ta* glands compared to wild-type controls (Fig. 4).

By contrast, *dl* SMGs are severely dysplastic. Like the *Ta* SMGs described above, the *dl* glands appear notably smaller than those of their wild-type littermates, with a significant 52% decrease ($t = 5.58$, $P < 0.05$) in total SMG protein level being detected. Histologically, *dl* SMGs present as a mass of undifferentiated epithelia with abnormal blood vessels and virtually no ducts, acini, or immunodetectable mucin protein (compare Fig. 5B with 5A, and Fig. 6B with 6A). Since no immunodetectable mucin protein was observed by using immunolocalization methodologies, we did not quantitate mucin protein level in *dl* mice.

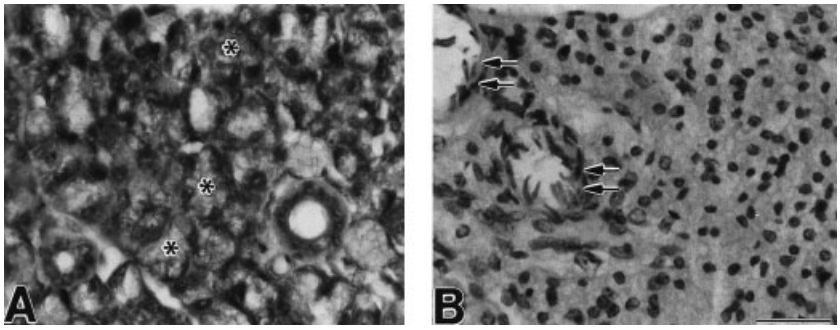

Fig. 5. *downless* (*dl*) SMGs are dysplastic. **A:** Control SMGs are characterized by differentiated ducts (double arrowheads) and acini (*). **B:** By contrast, no ducts or terminal buds are seen in *dl* ($Edar^{dl}/Edar^{dl}$) SMGs; rather, these SMGs appear as a mass of undifferentiated epithelial cells. Abnormal blood vessels (double arrows) are also seen in *dl* SMGs. It is important to note that strain differences account for the differences in SMG development in *dl* and *Ta* wild-type controls (Fig. 3A). Thus, the *dl* control SMGs appear younger than the *Ta* control SMGs. Bar = 50 μm.

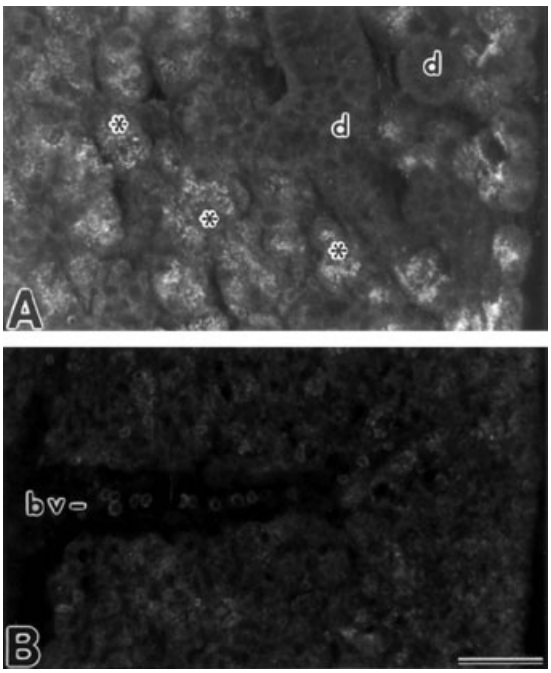

Fig. 6. Mucin protein is not immunodetected in dysplastic *downless* SMGs. Mucin protein was immunolocalized in (**A**) wild-type control SMG acini, but is virtually absent in (**B**) *dl* ($Edar^{dl}/Edar^{dl}$) SMGs. Moreover, one can clearly see SMG ducts (d) in wild-type, but not *dl*, glands. Abnormal blood vessels (bv) are also seen in *dl* glands. Note that strain differences account for the younger appearance and decrease in mucin protein expression in the *dl* control mouse compared to the *Ta* control. Bar = 50 μm.

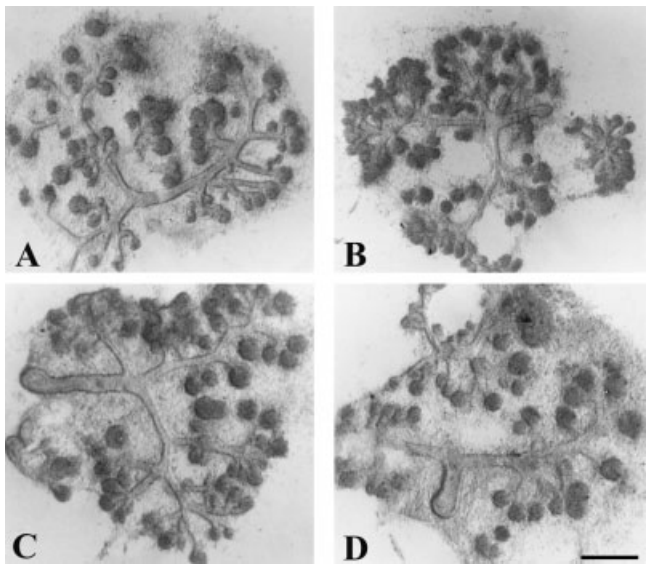

Fig. 7. Enhanced Eda/Edar signaling induces SMG branching morphogenesis, whereas abrogated signaling decreases branching morphogenesis. **A** and **B:** Paired E14 SMG primordia were cultured in the presence or absence of exogenous Eda ligand (0.5 μg/ml Flag-Eda-A1) for 3 days. (B) Eda supplementation induced a substantial increase in branch number compared to (A) control. **C** and **D:** Paired E14 SMG primordia were cultured in the presence or absence of 100 ng/ml hEdar-Fc chimera supplementation for 3 days, and a marked reduction in branch number is seen with (D) Edar-Fc supplementation compared to (C) control. (A similar reduction in branching, albeit to a greater extent, was observed with 150 ng/ml supplementation (data not shown).) Bar = 75 μm.

Enhanced and Interrupted Eda/Edar Signaling In Vitro

Given our observation of hypoplastic and dysplastic SMGs in mutant Eda^{Ta} (*Ta*) and $Edar^{dl}$ (*dl*) mice, respectively, we initiated in vitro mechanistic studies to clarify the role of Eda/Edar signal transduction in embryonic SMG development. First, we cultured embryonic Late Pseudoglandular stage (E14) SMGs for 3 days in the presence of exogenous Eda supplementation (Fig. 7), and assayed branching morphogenesis (Fig. 8). Since a notable difference in SMG branch number is seen among littermates, we compared the Spooner ratios in the right and left glands (treated and control) from each embryo by paired *t*-test for all embryos studied. Eda-supplemented explants exhibited a significant 28% increase ($t = 4.67$; $P < 0.01$) in epithelial branching compared to controls (Figs. 7 and 8).

Second, we used an in vitro inhibitory strategy to subvert the Eda/Edar signaling pathway. An exogenous

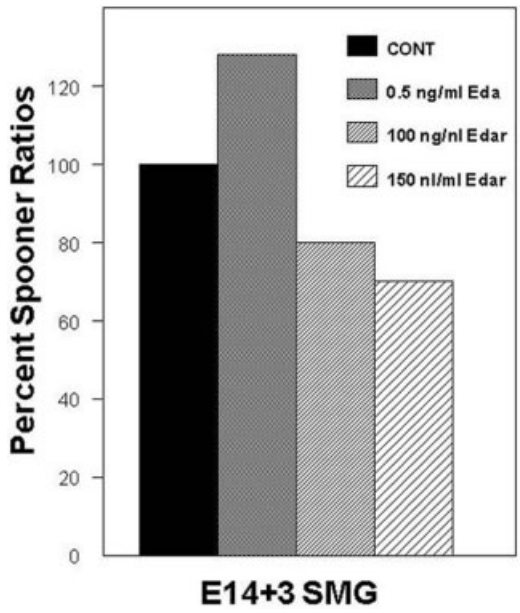

Fig. 8. Enhanced or abrogated Eda/Edar signaling induces significant changes in SMG branch number. Paired E14 SMG primordia were cultured in the presence or absence of exogenous Eda ligand (0.5 μg/ml Flag-Eda-A1), 100 ng/ml hEdar-Fc, or 150 ng/ml hEdar-Fc supplementation for 3 days. For each explant, the Spooner ratio (number of terminal bud number after 72 hr/initial bud number) was determined. The data were then arcsin-transformed and the means were compared by paired *t*-test as described in Materials and Methods. Eda supplementation induced a significant 28% increase ($t = 4.67$; $P < 0.01$) in branch number, whereas Edar-Fc (100 ng/ml or 150 ng/ml) supplementation induced a highly significant dose-dependent decrease (20% ($t = 5.58$, $P < 0.01$) and 30% ($t = 4.91$, $P < 0.01$), respectively) in branch number compared to controls.

Edar-Fc (100 or 150 ng/ml hEdar-Fc) chimera was added to the culture medium to competitively bind endogenous Eda and any other Edar ligands. This receptor/ligand binding methodology was previously used to interrupt tooth development in vitro (Tucker et al., 2000). When cultured with 100 ng/ml or 150 ng/ml soluble Edar, a highly significant dose-dependent decrease in branching (20% ($t = 5.58$, $P < 0.01$) and 30% ($t = 4.91$, $P < 0.01$), respectively) is seen compared to controls (Figs. 7 and 8). The difference between concentrations was also significant ($P < 0.05$).

Finally, given that overexpression of Eda or Edar has been shown to activate NF-κB in cell lines (Headon et al., 2001; Koppinen et al., 2001; Yan et al., 2000), we sought to determine whether enhanced Eda/Edar signaling in cultured embryonic SMGs induces NF-κB activation. Given that NF-κB is not immunodetected until the canalicular stage (Jaskoll and Melnick, 1999), and morphogenesis progresses markedly more slowly in vitro than in utero, we cultured E14 SMGs for 6 days to achieve the canalicular stage. Histological analysis of E14+6 SMGs demonstrated a late canalicular stage phenotype (data not shown). Moreover, since inactive NF-κB is found sequestered in the cytoplasm, whereas activated NF-κB is nuclear-localized (for review, see Senftleben and Karin, 2002), we assayed NF-κB activation by determining its spatial localization (cytoplasmic vs. nuclear) in control and Eda-supplemented explants cultured for 6 days. A notable increase in total immunodetectable and nuclear-localized (activated) NF-κB was seen with Eda supplementation compared to controls (compare Fig. 9B to 9A). This increase is similar to that seen with TNF supplementation (Melnick et al., 2001b).

DISCUSSION

Patients with HED exhibit SMG abnormalities (Clarke et al., 1987; Nordgarden et al., 1998), as well as absent or hypoplastic teeth, hair, and sweat glands (Gruneberg, 1965, 1971; Blecher et al., 1983; Clarke et al., 1987). Two mutant mouse strains, *Tabby* (*Ta*) and *downless* (*dl*), have phenotypes analogous to HED (Sunberg, 1994).The *Ta* (Eda^{Ta}/Y or Eda^{Ta}/Eda^{Ta}) mutant phenotype (Gruneberg, 1965, 1971; Sunberg, 1994) results from a truncated, loss-of-function Eda protein (Srivastava et al., 1997). Significantly, transgenic expression of the mouse Eda-A1 isoform in *Ta* rescues hair, sweat gland, and tooth development (Srivastava et al., 1997), indicating that this abnormal *Ta* phenotype is due to the lack of a functional Eda ligand. By contrast, the $dl^{Jackson}$ ($Edar^{dl}$) mutation is caused by the loss of a functional Eda receptor (Headon and Overbeek, 1999). With the demonstration that the mouse Eda and Edar genes are homologs of the human EDA and EDAR genes, respectively, as well as the presence of "HED-like" characteristics in *Ta* and *dl* mutant mice, we now have animal models in which to study Eda/Edar-mediated morphogenesis.

Previous studies of the *Ta* and *dl* mutant phenotypes primarily focused on tooth, hair, and sweat gland abnormalities (Gruneberg, 1965, 1971; Sofaer, 1969a, b; Headon and Overbeek, 1999; Pispa et al., 1999; Yan et al., 2000). Although the etiology of the *Ta* phenotype differs from that of the *dl* phenotype (i.e., absence of functional Eda ligand in *Ta* mice vs. absence of functional receptor in *dl* mice), their adult hair, tooth, and sweat gland phenotypes are indistinguishable (Grunberg, 1965, 1971; Sunberg, 1994). In this study, we analyzed the *Ta* and *dl* SMG phenotypes and demonstrated notable differences in them. *Ta* (Eda^{Ta}) SMGs are hypoplastic (Figs. 2 and 3), whereas *dl* ($Edar^{dl}$) SMGs are severely dysplastic (Figs. 4 and 5). These mutant-specific differences in SMG phenotype provide insight into the role of Edar-mediated signaling in SMG development. The absence of SMG ducts and acini in *dl* SMGs suggests that Eda/Edar signaling is essential for branching morphogenesis and lumina formation. Our localization of Eda and Edar proteins on SMG epithelia at sites of lumen formation, and on cells that are destined to undergo apoptosis (Jaskoll and Melnick, 1999) supports this conclusion. In addition, the significant decrease or absence of mucin protein in *Ta* and *dl* SMGs, respectively, indicates the importance of Eda/Edar signaling for glandular histodifferentiation. These results differ from those reported in a previous study of hair development (Laurikkala et al., 2002), in which Eda/Edar signaling was shown to regulate hair follicle morphogenesis but not epidermal cell differentiation. These reported differences between hair and SMG development are most likely due to tissue-specific roles for Eda/Edar signaling. Since the Eda ligand is present in *dl* mice, our finding of a *dl* SMGs phenotype suggests that no other receptor can compensate for the absence of Edar. Moreover, our observation of hypoplastic, not dysplastic, SMGs in *Ta* mice in the absence of a functional Eda-A1 ligand suggests that other

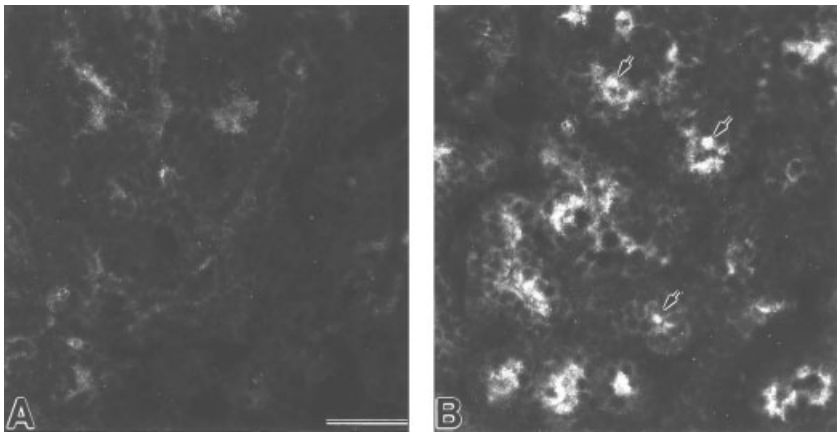

Fig. 9. Enhanced Eda/Edar signaling induces NF-κB activation. Embryonic SMG primordia were cultured for 6 days in the presence of (**A**) control medium or (**B**) 0.5 μg/ml Eda. Eda-supplemented explants exhibit a notable increase in overall immunodetectable NF-κB and in nuclear-localized (activated) NF-κB (arrows) compared to controls. Bar = 50 μm.

ligand(s) likely bind to Edar; a similar conclusion was reached by Tucker et al. (2000).

In addition, our finding of Eda and Edar proteins on epithelial cell membranes is consistent with previous reports that Eda and Edar are membrane proteins (Ferguson et al., 1997; Srivastava et al., 1997; Headon and Overbeek, 1999; Mikkola et al., 1999). A similar epithelial distribution pattern was seen during hair (Yan et al., 2000; Elomaa et al., 2001; Laurikkala et al., 2002) and tooth (Pispa et al., 1999; Tucker et al., 2000) development. This Eda epithelial localization is also consistent with the observation that the *Ta* mutant strain defect resides in the epithelium (Mayer and Green, 1978). Moreover, the codistribution of Eda and Edar primarily on SMG epithelia suggests that Eda/Edar likely acts in a juxtacrine manner, transducing its signal primarily within the epithelium. In an investigation of tooth and hair follicle development, Laurikkala et al. (2001, 2002) likewise concluded that Eda/Edar signaling mainly exerts its effect within the epithelium.

Our in vitro supplementation studies provide additional functional data in support of the notion that Eda/Edar plays an important role during embryonic SMG branching morphogenesis. The presence of exogenous Eda ligand induced a significant increase in branching, which indicates that enhanced Eda/Edar signaling induces branching morphogenesis. When the soluble form of Edar was added to the culture medium, a significant dose-dependent decrease in branching morphogenesis was seen. This result indicates that soluble Edar blocks endogenous Eda/Edar binding. However, abrogation with an Edar-Fc chimera produced a phenocopy of the *Ta* hypoplastic SMG, and not the *dl* dysplastic phenotype. A similar result was obtained by Tucker and coworkers (2000) in a study of tooth development. The most likely explanation for this phenotype is that the concentration and penetration of the soluble receptor into the explant was limited, and therefore not all of the endogenous Eda ligand was bound to the exogenous soluble Edar-Fc chimera. Our observation of a significant dose response supports this conclusion. Thus, not all Eda/Edar signaling was eliminated. Rather, it is likely that a small amount of endogenous ligand/receptor binding remained, resulting in a substantial decrease in (but not an absence of) Eda/Edar-mediated signaling and the ensuing hypoplastic gland. On the other hand, we cannot exclude the possibility that Edar may have ligand-independent activity, and that removal of the Eda ligand by a soluble receptor does not completely eliminate Edar signaling (Tucker et al., 2000). Further studies are needed to explain this phenotype.

Eda/Edar Signaling Induces NF-κB Activation

Gene-targeting experiments have demonstrated that Eda/Edar signaling activates NF-κB (Kumar et al., 2001; Doffinger et al., 2001; Headon et al., 2001; Koppinen et al., 2001). Moreover, mice with suppressed NF-κB/Rel exhibit defects in hair, exocrine gland, and tooth development identical to those seen in *Ta* and *dl* mutant mice (Schmidt-Ullrich et al., 2001). Of note, Doffinger et al. (2001) recently demonstrated the clinical importance of NF-κB for anhidrotic ectodermal dysplasia (HED). Impaired, but not abolished, NF-κB signaling resulted in X-linked recessive HED with immunodeficiency (HED-ID). HED-ID is associated with mutations in the IKBKG gene and not in the EDA and EDAR genes. IKBKG encodes IKKγ/NEMO, the regulatory unit of the IKK (IκB kinase) complex that is associated with an IKKα-IKKβ kinase heterodimer (for reviews, see Karin, 1999; Karin and Lin, 2002). IKKβ and IKKγ/NEMO are essential for NF-κB activation. Doffinger and colleagues (2001) have also shown that Edar-mediated signals induce NF-κB activation through the NEMO protein, which indicates that HED results from impaired NF-κB signaling. In the present study we have demonstrated that enhanced Eda/Edar signaling in vitro induces a notable increase in NF-κB activation; this increase in activated NF-κB is concomitant with a significant increase in branching morphogenesis. These results are consistent with our previous demonstration that NF-κB activation is essential for embryonic SMG cell cycle progression and epithelial cell proliferation (Melnick et al., 2001b). Inhibition of NF-κB nuclear translocation in vitro resulted in a smaller gland, which displayed a highly significant decrease in cell proliferation and a significant increase in apoptosis. Interruption of NF-κB activation during embry-

onic SMG development also resulted in a significant decrease in the expression of genes and proteins associated with cell cycle progression, and cell proliferation and survival. Given that patients with impaired NF-κB signaling exhibit HED-ID, *NF-κB/Rel* null mice exhibit "HED-like" hair, tooth, and glandular defects, and Eda/Edar signaling induces NF-κB activation in cell lines, our data suggest that the Eda/Edar/NF-κB pathway likely plays an essential role during embryonic SMG branching morphogenesis and histodifferentiation.

In conclusion, we investigated the role played by Eda/Edar signaling during embryonic SMG development by analyzing the spatiotemporal distribution of Eda and Edar proteins during that process, and assessing *Ta* and *dl* mutant mouse SMGs. Our localization of Eda and Edar proteins at sites of lumen formation and on cells destined to undergo apoptosis, and our observations of a hypoplastic SMG phenotype in *Ta* mice and the absence of ductal and acinar lumina and acinar cell histodifferentiation in *dl* SMGs, suggest that the Eda/Edar signal transduction pathway plays an essential role in SMG epithelial lumina formation and histodifferentiation. Moreover, the presence of SMGs in both *Ta* and *dl* mutant mice, as well as the absence of immunodetectable Eda and Edar protein in Initial Bud and Early Pseudoglandular stage SMGs, indicate that Eda/Edar-mediated signaling is important for branching morphogenesis but not for initial gland formation. Finally, the results of our in vitro experiments, together with the analysis of *Ta* and *dl* SMGs, suggest that Eda/Edar/NF-κB signaling regulates epithelial cell proliferation, survival, and histodifferentiation. The precise molecular details regarding the downstream targets of Eda/Edar/NF-κB signaling must be addressed in future studies.

ACKNOWLEDGMENTS

We thank Genentech for generously providing the Eda-A1 and Edar-Fc used in this study.

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