

Submandibular Gland Morphogenesis: Stage-Specific Expression of TGF- α /EGF, IGF, TGF- β , TNF, and IL-6 Signal Transduction in Normal Embryonic Mice and the Phenotypic Effects of TGF- β 2, TGF- β 3, and EGF-R Null Mutations

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ABSTRACT

Branching morphogenesis of the mouse submandibular gland (SMG) is dependent on cell-cell conversations between and within epithelium and mesenchyme. Such conversations are typically mediated in other branching organs (lung, mammary glands, etc.) by hormones, growth factors, cytokines, and the like in such a way as to translate endocrine, autocrine, and paracrine signals into specific gene responses regulating cell division, apoptosis, and histodifferentiation. We report here the protein expression in embryonic SMGs of four signal transduction pathways: TGF- α /EGF/EGF-R; IGF-II/IGF-IR/IGF-IIR; TGF- β s and cognate receptors; TNF, IL-6, and cognate receptors. Their *in vivo* spatiotemporal expression is correlated with specific stages of progressive SMG development and particular patterns of cell proliferation, apoptosis, and mucin expression. Functional necessity regarding several of these pathways was assessed in mice with relevant null mutations (TGF- β 2, TGF- β 3, EGF-R). Among many observations, the following seem of particular importance: (1) TGF- α and EGF-R, but not EGF, are found in the *Initial* and *Pseudoglandular Stages* of SMG development; (2) ductal and presumptive acini lumina formation was associated with apoptosis and TNF/TNF-R1 signalling; (3) TGF- β 2 and TGF- β 3 null mice have normal SMG phenotypes, suggesting the presence of other pathways of mitostasis; (4) EGF-R null mice displayed an abnormal SMG phenotype consisting of decreased branching. These and other findings provide insight into the design of future functional studies. Anat Rec 256:252–268, 1999.

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Key words: embryonic submandibular gland; TGF- β ; TNF; IGF-II; TGF- α /EGF; IL-6

Branching morphogenesis is fundamental to many developing organs: salivary gland, lung, mammary gland, pancreas, and kidney (see review, Wessells, 1977). Branching organs achieve tree- or bush-like morphology through a program of repetitive, self-similar furcations that serve as branch points for new epithelial outgrowths (Spooner et al., 1989; Mandelbrot, 1983). Branching morphogenesis of the mouse submandibular gland (SMG) is dependent on

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epithelial-mesenchymal interactions, with the mesenchyme being permissive or instructive (Wessells, 1977; Nakanishi et al., 1987; Cutler and Gremski, 1991). Almost certainly, the cellular conversation occurs not only between epithelium and mesenchyme, but within these tissues as well. Such conversations in other branching organs are typically mediated by hormones, growth factors, cytokines, and the like in such a way as to translate endocrine, autocrine, and paracrine signals into specific gene responses regulating cell division, apoptosis, and histodifferentiation (Jaskoll et al., 1994a, 1996; Melnick et al., 1996; Miettinen et al., 1997; Zhao et al., 1998). Relatively little is known about this in SMGs.

These signal transduction pathways comprise a complex network of mutually dependent organizing mechanisms responsible for progressive differentiation of the multicellular organ. Not all encoded signal proteins and their downstream effectors are expressed at any given point in time and space. Rather, the pattern is dynamic and changes with the stage of development. In this paper, we report the unique protein expression in embryonic SMGs of four signal transduction pathways: TGF- α /EGF/EGF-R; IGF-II/IGF-IR/IGF-IIR; TGF- β s and cognate receptors; TNF, IL-6, and cognate receptors. Their *in vivo* spatiotemporal expression is correlated with specific stages of progressive SMG development, as well as stage-specific patterns of cell proliferation, apoptosis, and mucin protein expression. Functional necessity regarding several of these pathways is assessed in mice with relevant null mutations (TGF- β 2, TGF- β 3, EGF-R). What emerges is a rational framework for future multifactorial functional studies of related signaling phenomena (cellular networking motifs).

MATERIALS AND METHODS

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Melnick et al., 1998); plug date = 0 of gestation. Pregnant females were anesthetized on days 14–18 of gestation with methoxyflurane (metafane) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler (1989). SMGs were dissected, fixed in Carnoy's fixative, processed, embedded in low-melting point paraplast, and stored for brief periods at 4°C as previously described (Melnick et al., 1998). A minimum of three litters were collected for each developmental stage.

Immunolocalization

The tissues were sectioned at 5 μ m, placed on cleaned, gelatin-coated slides at 37°C for 3 hr, and immediately immunostained as previously described (Melnick et al., 1998). For rabbit polyclonal antibodies (anti-IGF-IIR, IGF-IR, TGF- β -RI, TGF- β -RII, TGF- β 1, TGF- β 2, EGF-R, IL-6R), the sections were incubated overnight with the primary antibody and then sequentially incubated in biotin-labelled goat anti-rabbit IgG (Organon Teknika, Durham, NC) and FITC-labelled streptavidin (Zymed, South San Francisco, CA). For goat polyclonal antibodies (anti-TGF- β 3, TGF- α , EGF, TNF- α , TNF-R1, TNF-R2, IL-6), the sections were incubated overnight with the primary antibody and then incubated with FITC-labelled anti-goat IgG (Sigma, St. Louis, MO). In all experiments, control sections were incubated in the absence of primary

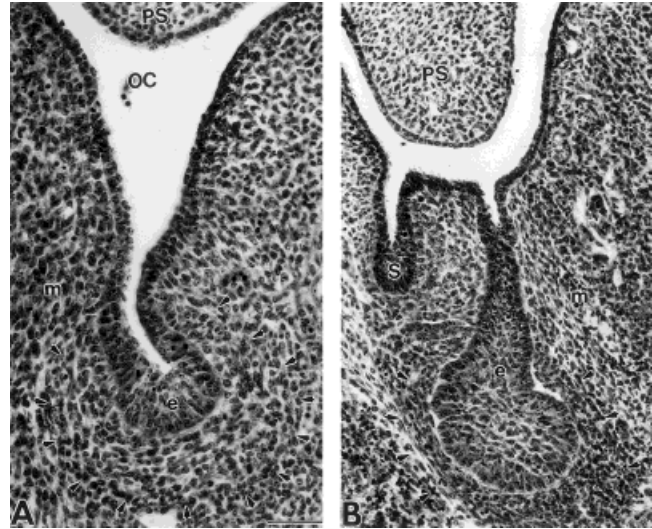


Fig. 1. Early embryonic SMG morphogenesis. **A:** *Early Initial Bud Stage:* the oral epithelium on the floor of the oral cavity (OC) proliferates to form a thickened epithelium (e) which grows down into a mesenchymal condensation (arrowheads) as the initial SMG bud. **B:** *Late Initial Bud Stage:* due to continued epithelial cell proliferation and down-growth into the condensed mesenchyme (arrowheads), the SMG primordium appears as an elongated, solid epithelial stalk with a terminal end-bulb. It is interesting to note that the initial stage of sublingual gland (S) formation is similar to that seen for the SMG. PS, palatal shelves; m, mesenchyme. Scale bar = 50 μ m.

antibody or with preimmune serum; controls were routinely negative. For evaluation of IGF-II, a monoclonal antibody (Mab) was used; these sections were preincubated with unlabelled goat anti-mouse IgG to block non specific binding of the secondary antibody to mouse IgGs prior to overnight incubation with anti-IGF-II. Sections were then sequentially incubated in biotin-labelled goat anti-mouse IgG (Organon Teknika) and FITC-labelled streptavidin. Controls were incubated in the absence of the primary Mab. In all experiments, the controls were routinely negative. A minimum of three SMGs per strain were evaluated for each stage of development.

Antibodies

Anti-IGF-IIR antiserum (antiserum # 3637) was kindly provided by Dr. Peter Nissley. Polyclonal antibodies to IGF-II, IGF-IR α , IL-6, IL-6R, TGF- β -RI, TGF- β -RII, TGF- β 1, TNF α , TNF-R1, TNF-R2, TGF- α , EGF, and EGF-R were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Anti-TGF- β 2 and anti-TGF- β 3 polyclonal antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-rat IGF-II monoclonal antibody was purchased from Amano Enzyme (Lombard, IL).

Proliferation Assay

Sections were incubated with anti-PCNA using the Zymed mouse PCNA kit and then counterstained with hematoxylin for 4 min. Sections were evaluated by routine light microscopy.

Apoptosis Assay

Apoptotic cells were detected using a monoclonal antibody to single-stranded DNA (ssDNA) (Mab F7–26) accord-

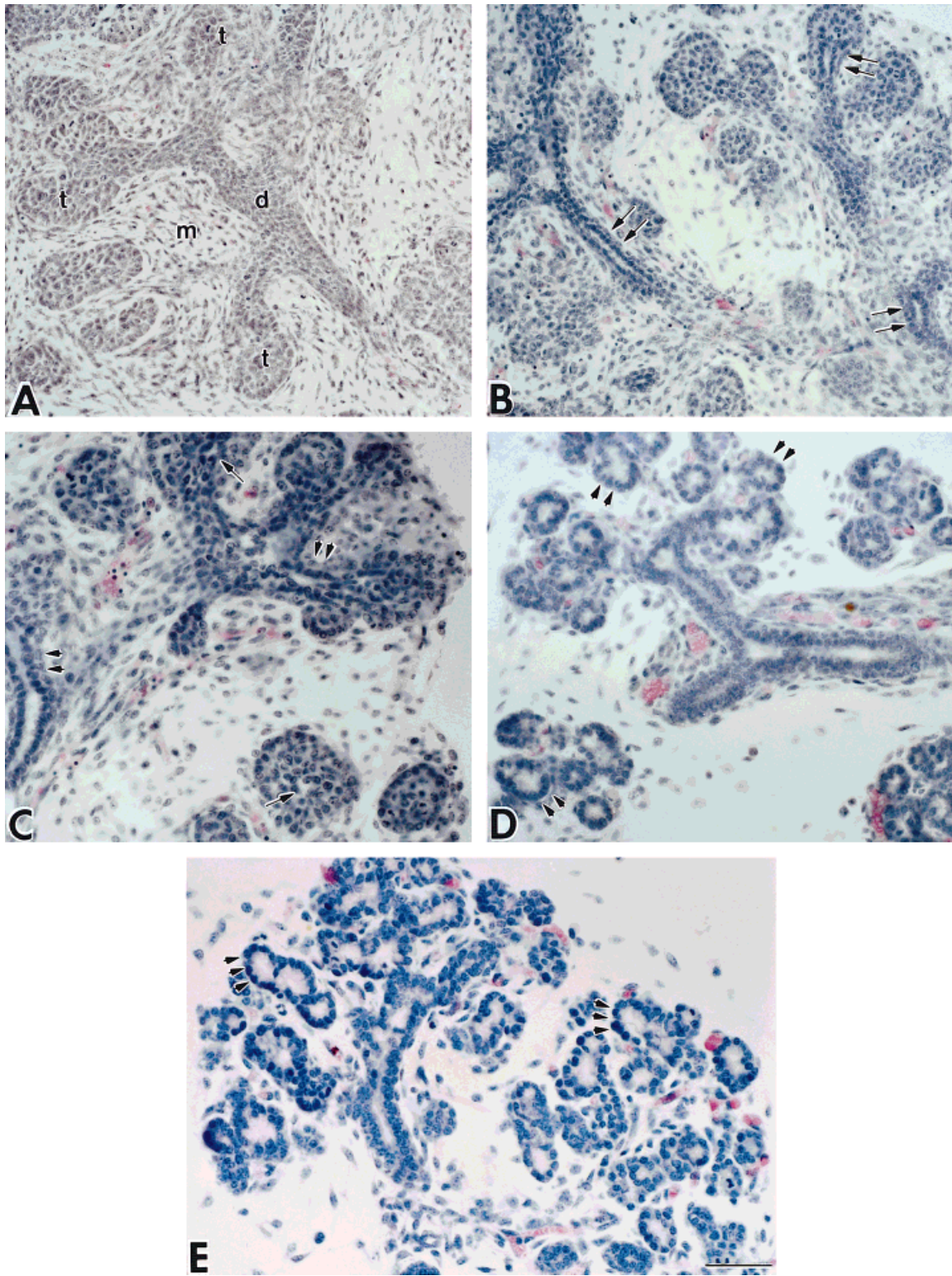


Fig. 2. Subsequent stages of embryonic SMG development. **A:** In the *Pseudoglandular Stage*, the compact, solid cord of epithelia elongates to form the presumptive ducts (d) and terminal end bud (t). This branching epithelium is surrounded by closely-packed mesenchyme (m). **B:** In the *Early Canalicular Stage*, there is a substantial increase in the number of epithelial lobes due to branching morphogenesis. The ductal and terminal bud epithelia are surrounded by more loosely-packed mesenchyme. The ducts consists of a multilayer of cuboidal cells (double arrows) and begin to exhibit distinct lumena; no lumena are seen in the terminal end buds. **C:** By the *Late Canalicular Stage*, a bilayer of cuboidal cells surrounds the

larger ductal lumena (double arrowheads). The terminal end buds primarily consist of loosely-packed epithelial cells; lumena are just beginning to form in the end buds (arrowhead). **D:** By the *Early Terminal Bud Stage*, clusters of terminal end buds exhibit distinct lumena surrounded by cuboidal cells (double arrowheads). At this stage, ductal and terminal bud lumena are starting to become contiguous. **E:** By the *Late Terminal Bud Stage*, the terminal bud lumena have increased in size and many terminal end bud lumena are now continuous with ductal lumena. Duct and terminal end buds consist of an single layer of cuboidal cells (triple arrowheads) lining the lumena. Scale bar = 50 μ m.

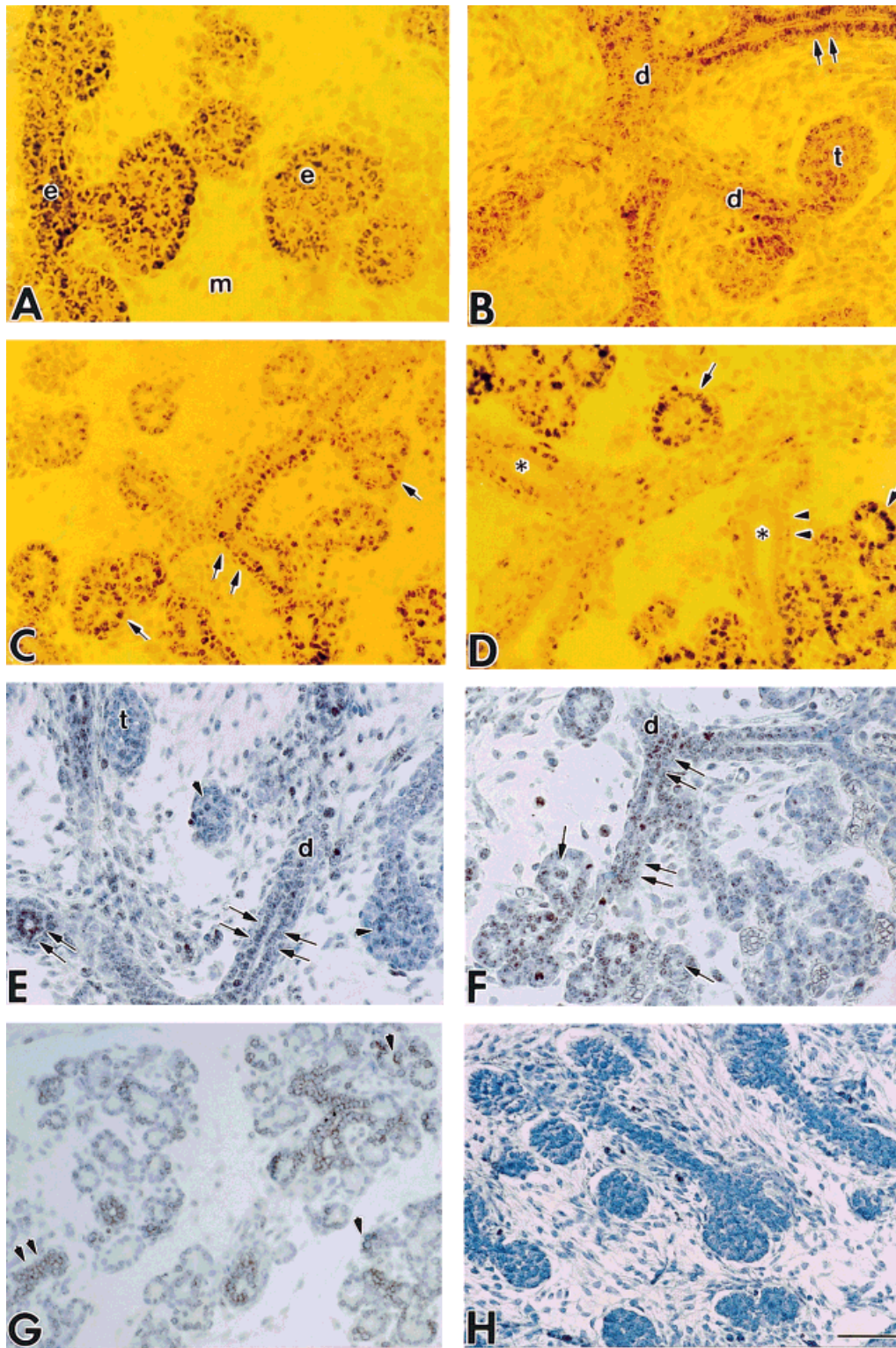


Fig. 3. Embryonic SMG cell proliferation. **A:** In the *Pseudoglandular Stage*, cell proliferation is seen throughout the branching epithelia (e) but is absent in the mesenchyme (m). **B:** In the *Canalicular Stage*, presumptive ductal (d) and terminal end-bud (t) epithelia exhibit extensive cell proliferation. Intense immunostain in the cuboidal cells facing the ductal lumina (double arrows) is seen. **C:** In the *Early Terminal Bud Stage*, proliferating cells are primarily seen in terminal end bud (arrow) and ductal (double arrows) epithelia. **D:** By the *Late Terminal Bud Stage*, cell proliferation is primarily seen in terminal end bud epithelia (arrow), as well as in mesenchymal cells (double arrowheads) adjacent to the basal surfaces of ductal epithelia. A decrease in cell proliferation is found in ductal cuboidal cells surrounding larger lumina (*). **E–H:** Programmed cell death. **E:** In the *Canalicular Stage*, small lumina are seen in the developing ducts (d); at the sites where lumina are forming, apoptotic nuclei are detected (double arrows). Since lumen are not present in

terminal end buds at this stage, only a few apoptotic nuclei (arrowhead) are seen in regions where the lumen will later develop. **F:** By the *Early Terminal Bud Stage*, there is a marked increase in ductal lumen size, with several terminal end buds now exhibiting lumina. At sites of lumen formation, apoptotic nuclei are detected. An increase in apoptosis is observed in the terminal end bud epithelia, with a decrease being seen in ductal epithelia with distinct lumen. Apoptotic nuclei can be seen in closed ducts where a lumen will later form (double arrows); isolated apoptotic nuclei can also be seen with a few lumina (arrow). **G:** By the *Late Terminal Bud Stage*, apoptotic cells are detected at sites where ductal and terminal end bud lumina are becoming contiguous (double arrowheads). Apoptosis is also observed in terminal end bud epithelia (arrowhead), although to a lesser degree than in the *Early Terminal Bud Stage*. **H:** A negative control section of *Early Canalicular Stage* pretreated with lysine-rich histone. Scale bar = 50 μm.

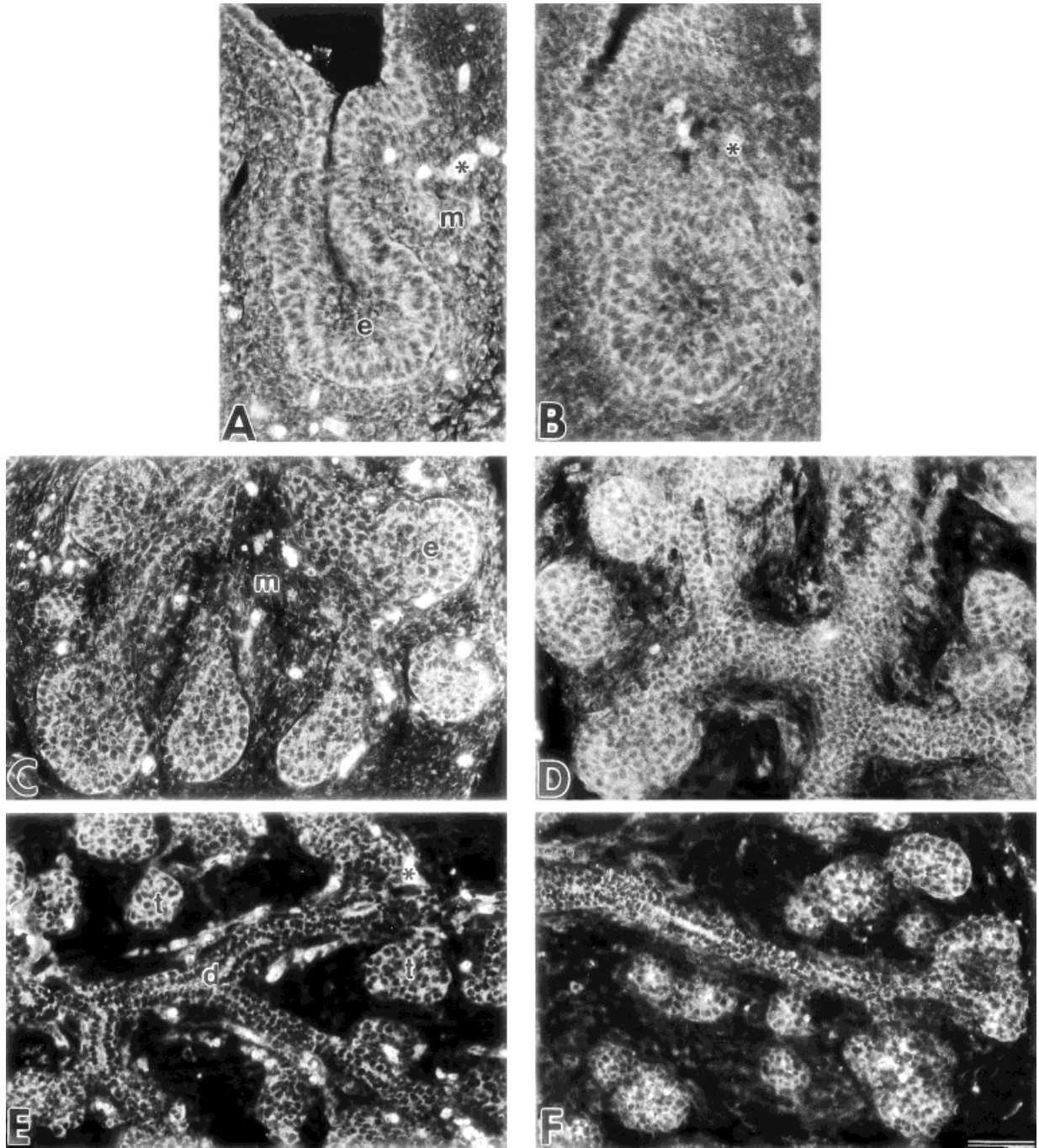


Fig. 4. Immunolocalization of TGF- α , EGF, and EGF-R. A,B: *Initial Bud Stage*. TGF- α (A) and its receptor EGF-R (B) are primarily immunolocalized in the epithelial stalk and bulb, as well as in the condensed mesenchyme. C,D: *Pseudoglandular Stage*. TGF- α (C) and EGF-R (D) are mostly localized in the branching epithelia (e), with little seen in the mesenchyme (m). E,F: *Early Canalicular Stage*. TGF- α (E) is seen on ductal (d) and terminal end bud (t) epithelia; TGF- α is also seen in blood vessels (*). EGF (F) is detected for the first time at this stage, within the developing ducts (d) and terminal end buds (t). G-I: *Late Canalicular Stage*. TGF- α (G) is primarily immunolocalized on the ductal epithelia lining the lumen (arrowheads); EGF (H) is primarily immunodetected in the center of terminal end buds and in ductal epithelia cell surrounding

formed lumina (double arrowheads); and EGF-R (I) is distributed throughout ductal and terminal bud epithelia. J-L: *Terminal Bud Stage*. TGF- α (J) retains its ductal distribution seen in the *Late Canalicular Stage*. However, there is a considerable decrease in TGF- α immunostain in terminal end bud epithelia surrounding large lumina (double arrows). EGF (K) is localized in the central regions of the terminal end buds (arrows) as well as on the outer ductal epithelium (triple arrowheads). A decrease in EGF immunostain is seen in terminal buds with lumina (double arrows). EGF-R (L) is primarily immunolocalized on ductal epithelia; weak EGF-R immunostain is also observed on terminal end bud epithelia (arrow head). Note that TGF- α and EGF-R are also detected in blood vessels (*). Scale bar = 25 μ m in A,B; 50 μ m in C-L.

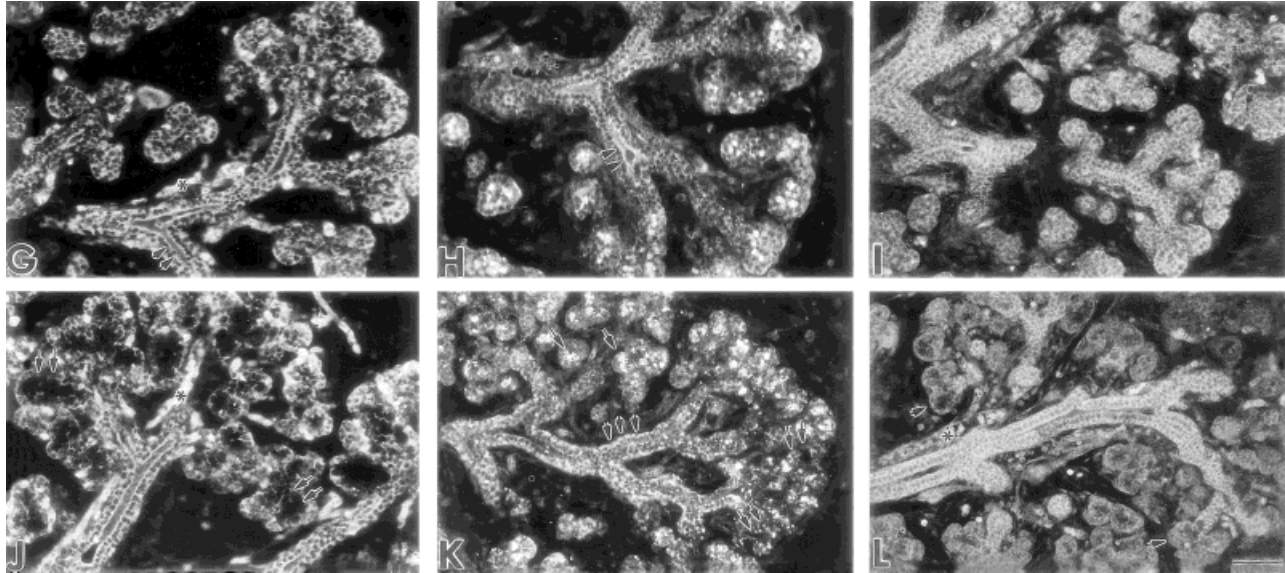


Figure 4. (Continued)

ing to the method of Apostain, Inc. (Miami, FL). Selective binding to anti-ssDNA monoclonal antibody F7-26 to apoptotic nuclei reflects decreased stability of DNA to thermal denaturation. Decreased stability of apoptotic DNA toward thermal denaturation is induced by proteolysis of DNA-bound proteins during apoptosis. The procedure consists of two main steps: (1) tissue sections are heated at conditions inducing DNA denaturation *in situ* only in apoptotic nuclei; and (2) single-stranded regions in apoptotic DNA (ssDNA) are recognized with Mab F7-26 previously shown to be highly specific to DNA in single stranded conformation (Frankfurt et al., 1996). The higher sensitivity of Mab staining compared to TUNEL is due to the different mechanisms of the two techniques. TUNEL detects internucleosomal DNA fragmentation associated with late apoptosis, while Mabs to ssDNA detect the early stages of apoptosis and stain apoptotic nuclei in the absence of low molecular weight DNA fragmentation (Frankfurt et al., 1996). Importantly, in contrast to the TUNEL method, monoclonal antibodies to ssDNA have been shown to be specific for apoptotic cell death and does not detect necrotic cells. Four positive and negative controls were conducted.

Negative controls. (1) Tissue sections were heated and treated with S1 nuclease (Sigma); S1 nuclease eliminates staining of apoptotic cells, thus demonstrating that Mab F7-26 binds specifically to ssDNA. (2) Sections were pretreated in PBS containing lysine-rich histone (Sigma) prior to heating and immunostaining; reconstitution with histone restores DNA stability in apoptotic nuclei, thus preventing DNA denaturation and eliminating Mab staining of apoptotic cells.

Positive controls. (1) Sections were heated in water and treated with Mab; bright staining of all non-apoptotic nuclei with low apoptotic indexes demonstrates that the procedure is adequate to detect ssDNA. (2) Sections were pretreated with proteinase K before heating; intensive staining of non-apoptotic cells demonstrates the procedure detects decreased DNA stability induced by the

digestion of nuclear proteins. Mab F7-26 was purchased from Apostain, Inc.

Evaluation of TGF- β 2, TGF- β 3, and EGF-R Null Mice

E18.5 TGF- β 2 null (-/-) and wildtype mice were obtained from Dr. Doetschman (Sanford et al., 1997). E16.5 and newborn TGF- β 3 null (-/-) and wildtype mice were obtained from Dr. Groffen (Kaartinen et al., 1995). Newborn EGF-R null (-/-) and wildtype mice were obtained from Dr. Miettinen (Miettinen et al., 1995). The submandibular glands were dissected, embedded in paraffin, sectioned at 7 μ m, stained with hematoxylin and eosin, and examined by conventional light microscopy.

RESULTS

The submandibular gland (SMG) begins as a outgrowth of the oral epithelium into the underlying mandibular mesenchyme on E11.5 in B10.A mice (Fig. 1A). The initial SMG bud elongates to form a solid epithelial cord with a bulb at its distal end (Fig. 1B). Clefs subdivide the initially solid epithelial bulb to begin the branching process (Wessells, 1977). Repeated branching of the SMG epithelial buds transform the embryonic SMG into a "bush-like" structure which consists of clusters of terminal end buds connecting to the ductal system. Because the embryonic SMG develops by repeated epithelial end bud branching, the morphogenetic state of terminal bud clusters differs between SMG regions, dependent on the time of branch formation. Thus, it is more informative to speak of developmental stage than gestational age.

Stages of Embryonic SMG Development

To enable the mapping of important signal transduction molecules during embryonic SMG development, we first staged the developing SMG and evaluated cell proliferation and apoptosis (Figs. 1-3). In the *Initial Bud Stage*, proliferation of the primitive oral cavity epithelium adja-

cent to the developing tongue produces a thickened epithelium which grows down into a mesenchymal condensation to form the initial SMG bud (Fig. 1A). Continued epithelial cell proliferation and downward growth results in the SMG primordium becoming a solid, elongated epithelial stalk terminating in a bulb (Fig. 1B). During this initial stage of SMG development, cell proliferation is seen throughout the epithelium and apoptosis is absent (data not shown). In the *Pseudoglandular Stage*, the solid cord of epithelia elongates and grows by repeated end-bud branching into the surrounding mesenchyme (Fig. 2A). Cell proliferation is seen throughout the branching epithelia whereas it is absent from the mesenchyme (Fig. 3A). At this stage, no ductal lumen is seen. With progressive development: (1) SMG epithelia continue to elongate and branch into the more loosely-packed mesenchyme to become the dominant element of the gland, and (2) mesenchymal cells move further apart, with the space between mesenchymal cells being occupied by extracellular matrix (ECM) components such as laminin, collagen, and fibronectin (Cutler, 1990; Hardman and Spooner, 1992; Macauley et al., 1997).

By the *Early Canalicular Stage* (Fig. 2B), the number of epithelial lobes has increased, the presumptive ducts begin to exhibit distinct lumina lined by cuboidal epithelial cells, and the mesenchyme becomes more loosely packed. Cell proliferation is detected throughout ductal and terminal end-bud epithelia (Fig. 3B). At sites of lumen formation in the developing ducts, many apoptotic nuclei are seen (Fig. 3E). Since lumina are just beginning to form in the terminal end buds at this stage of development, only a few apoptotic cells are seen within the end bud epithelium. By the *Late Canalicular Stage* (Fig. 2C), a distinct bilayer of cuboidal cells surround the ductal lumina. The patterns of cell proliferation and programmed cell death is similar to that seen in the *Early Canalicular Stage* (data not shown).

The *Early Terminal Bud Stage* (Fig. 2D) is characterized by clusters of terminal end buds with distinct lumina surrounded by one or more layers of cuboidal cells. At this stage, mucin protein is first immunodetected (Jaskoll et al., 1998). Cell proliferation is primarily seen in terminal end bud and ductal epithelia (Fig. 3C). In the ductal and terminal end buds where lumina are forming, numerous apoptotic nuclei are readily seen (Fig. 3F); a few apoptotic cells can also be seen within a few lumen and in closed ducts. Amorphous material can be seen within several lumina (Fig. 2D); this material may contain "secretory proteins" previously identified in the prenatal SMGs by Ball and coworkers (Ball et al., 1991; Moreira et al., 1991), as well as debris from apoptotic cells. By the *Late Terminal Bud Stage* (Fig. 2E), continuity between terminal end bud lumina and ductal lumina is common but not complete. Apoptosis is also seen in terminal end bud epithelia, although to a lesser degree than in the *Early Terminal Bud Stage* (compare Fig. 3F to G). Ducts and terminal end buds are now distinguished by more mature lumina consisting of a single layer of epithelial cells with mesenchymal cells often adjacent to their basal surfaces (basement membranes; Figs. 2E, 3D). Since the terminal bud lumina continue to enlarge, cell proliferation is primarily seen in terminal end bud epithelia; proliferation is nearly absent from ductal epithelia (Fig. 3D). At this stage, mucin is expressed intracellularly in numerous terminal end buds (Jaskoll et al., 1998).

TGF- α /EGF/EGF-R

The EGF receptor (EGF-R) signal transduction pathway has been shown to promote *in vitro* cell proliferation and branching morphogenesis in embryonic salivary glands (Nogawa and Takahashi, 1991; Kashimata and Gresik, 1997). Ligands EGF and TGF- α both bind to EGF-R to transduce the growth signal (Partenen, 1990). Although EGF and TGF- α transcripts have been detected in early embryonic SMGs (Kashimata and Gresik, 1997) and the spatial distribution of EGF-R protein has recently been described in fetal SMGs (Gresik et al., 1997), little is known about the relationship between the spatiotemporal distribution of ligand (EGF or TGF- α) relative to receptor. TGF- α and EGF-R, but *not* EGF, are immunolocalized to the cell surfaces of SMG epithelial bud and adjacent mesenchyme in the *Initial Bud Stage* (Fig. 4A,B). Assuming that they are both functional, this would suggest that TGF- α is EGF-R's ligand at this early stage of SMG development.

By the *Pseudoglandular Stage* (Fig. 4C,D), TGF- α and EGF-R are mostly seen in the branching epithelia and far less in the mesenchyme. The *Early Canalicular Stage* is the stage in which EGF is first immunodetected: EGF and TGF- α are immunolocalized in SMG epithelia (Fig. 4E,F), with EGF immunostain being most intense within the center of the terminal end buds (Fig. 4F); EGF-R epithelial distribution is unchanged (data not shown). In the *Late Canalicular Stage* (Fig. 4G-I), TGF- α is newly localized on the ductal epithelial surfaces facing the lumen (Fig. 4G); EGF is primarily localized to epithelial cells in the center of terminal end buds and in ductal cells surrounding the lumen (Fig. 4H); and EGF-R is distributed throughout ductal and terminal end bud epithelia (Fig. 4I).

By the *Terminal Bud Stage*, clear differences in the cell-specific distribution of TGF- α and EGF are seen (Fig. 4J-L): TGF- α is most prominently immunolocalized in ductal epithelia (Fig. 4J) and EGF is prominent in the center of terminal end buds and on ductal epithelia (Fig. 4K). EGF-R continues its more ubiquitous distribution in the *Terminal Bud Stage*, being immunodetected in ductal epithelia and, to a lesser extent, in terminal end bud epithelia (Fig. 4L). This pattern of EGF-R immunolocalization is similar to that seen by Gresik et al. (1997). Our results suggest that the growth signal is mediated in early branching morphogenesis by the binding of TGF- α to EGF-R and that later growth is promoted by both TGF- α /EGF-R and EGF/EGF-R binding, although in distinctly different cell populations.

EGF-R Null Mice

Given that functional EGF-R appears to be an important regulator of SMG branching morphogenesis *in vitro* (Kashimata and Gresik, 1997) and that EGF-R null mice exhibit abnormal lung branching *in vivo* (Miettinen et al., 1997), we postulated that EGF-R is essential for normal *in vivo* SMG branching morphogenesis. To address this hypothesis, we evaluated neonatal EGF-R null mice. While there was no apparent macroscopic differences from wildtype upon dissection, microscopic examination revealed a very substantial reduction in terminal buds per unit area, accompanied by an increase in extracellular matrix (Fig. 5). There were no differences in the degree of maturity (stage) of the terminal buds between null and wildtype neonatal mice (compare Fig. 5D and B). This suggests that

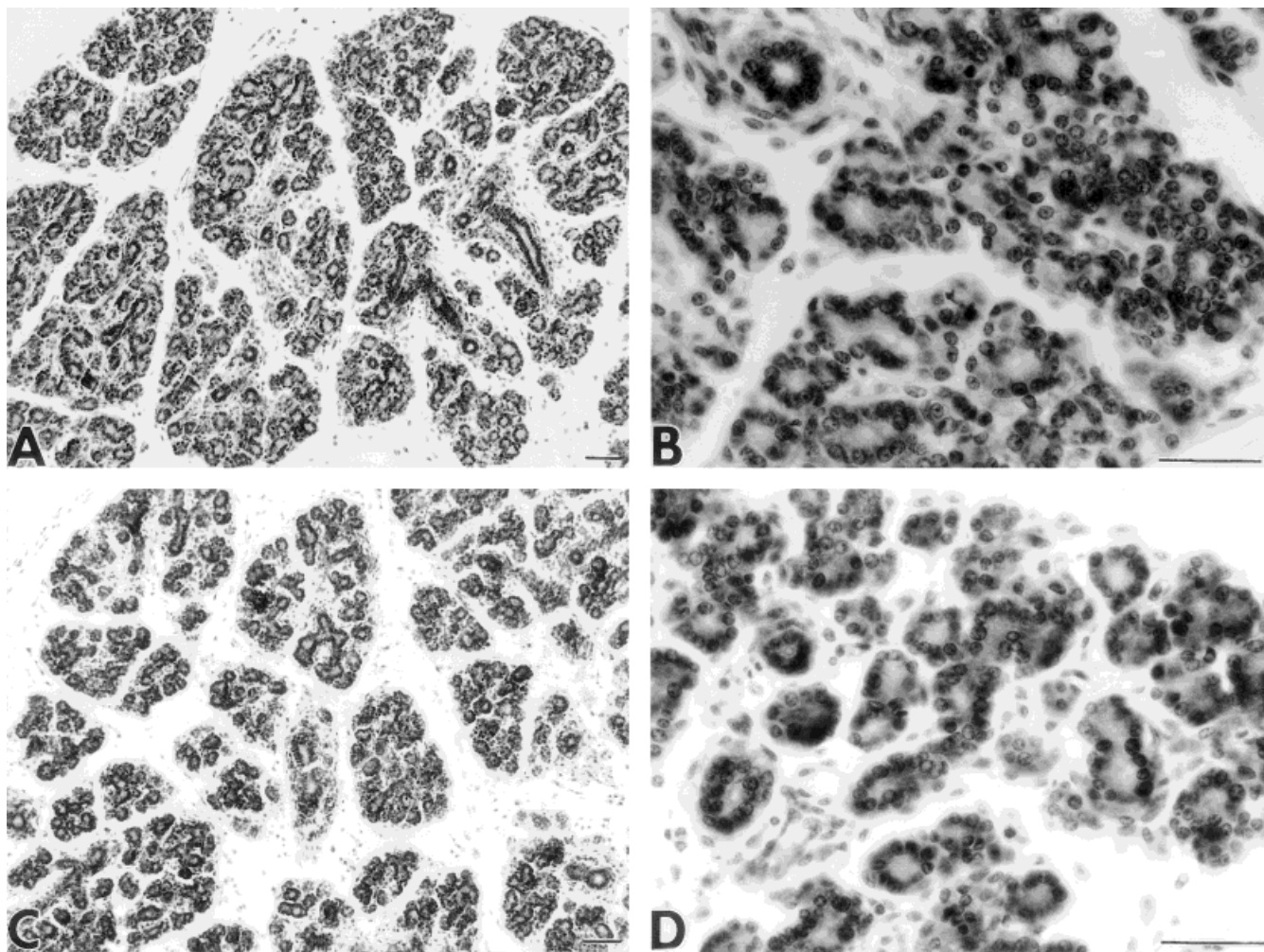


Fig. 5. Submandibular gland morphology in EGF-R null mice. **A,B:** Newborn EGF-R $+/+$ SMG. **C,D:** Newborn EGF-R $-/-$ SMG. Although the overall size of the SMGs are similar in EGF-R null and wildtype mice, EGF-R null mouse SMGs exhibit a substantial decrease in epithelial branch number compared to wildtype glands. Higher magnification of

EGF-R $+/+$ (B) and EGF-R $-/-$ (D) SMGs demonstrates the similarity in the degree of glandular histodifferentiation in EGF-R null and wildtype SMGs, both staging as *Late Terminal Bud*. Scale bar = 50 μ m in A,C; 35 μ m in B,D.

the EGF-R pathway is an important regulator of SMG branch number *in vivo*, but is not essential for SMG initiation or maturation.

IGF-II/IGF-IR/IGF-IIR

The growth factor IGF-II binds to two receptors, IGF-IR and IGF-IIR (Barlow, 1995). IGF-II binding to the type 1 receptor (IGF-IR) mediates the growth signal whereas IGF-II/IGF-IIR (type 2 receptor) binding does not transduce mitogenic signals; IGF-II binding to IGF-IIR sequesters IGF-II from IGF-IR, thereby decreasing the levels of IGF-II available for growth promotion (see reviews Baker et al., 1993; Barlow, 1995; Haig and Graham, 1991). IGF-II, and its two receptors, IGF-IR and IGF-IIR, are seen in the *Initial Bud Stage*: IGF-II, IGF-IR, and IGF-IIR are localized throughout the SMG epithelium and surrounding mesenchyme (Fig. 6A–C). In the *Pseudoglandular Stage*, IGF-II and IGF-IR are primarily localized throughout the epithelia (Fig. 6D,F) whereas IGF-IIR is

more uniformly distributed throughout the epithelia and mesenchyme (Fig. 6H). By the *Late Canalicular/Early Terminal Bud Stage*, IGF-II is immunolocalized on the outer epithelial surfaces of terminal end buds, and to a lesser extent, throughout the mesenchyme (Fig. 6E); IGF-IR is primarily immunodetected in ductal epithelia and weakly detected in bud epithelia (Fig. 6G); and IGF-IIR is primarily localized throughout the mesenchyme, and to a lesser extent, on the outer and inner ductal epithelial surfaces (Fig. 6I). A similar pattern of IGF-II, IGF-IR and IGF-IIR spatial distribution persists into the *Late Terminal Bud Stage* (data not shown).

TNF, IL-6, and Cognate Receptors

We have previously demonstrated that TNF is important to embryonic pulmonary branching morphogenesis (Jaskoll et al., 1994a; Melnick et al., 1996). Thus, we in-

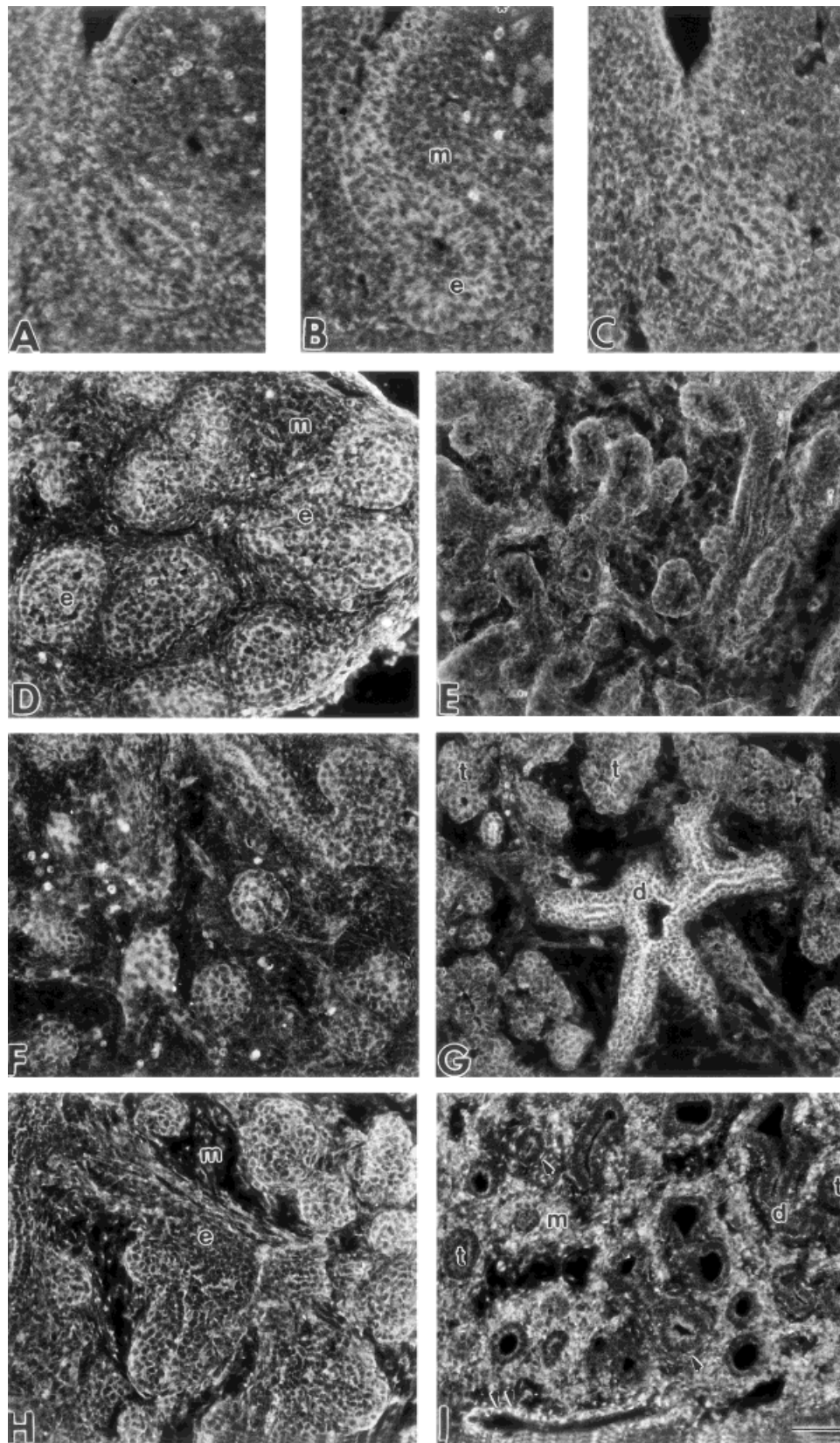


Fig. 6. Immunolocalization of IGF-II, IGF-IR and IGF-IIR. A–C: *Initial Bud Stage*. IGF-II (A), IGF-IR (B), and IGF-IIR (C) are immunolocalized in both epithelium (e) and mesenchyme (m). D, F, H: *Pseudoglandular Stage*: IGF-II (D) and IGF-IR (F) are mostly localized throughout the epithelia, and to a lesser extent, in the mesenchyme. By contrast, IGF-IIR (H) is distributed more uniformly throughout the epithelia and mesenchyme. E, G, I: *Canalicular Stage*: IGF-II (E) is localized on the basolateral epithelial surfaces, and to a lesser extent, throughout SMG mesenchyme

and epithelium. IGF-IR (G) is immunolocalized in ductal (d) epithelia, and weakly so in terminal end buds (t). IGF-IIR (I) is primarily localized throughout the mesenchyme and on epithelial surfaces (arrows), with IGF-IIR immunostain being seen in mesenchymal cells surrounding the ducts (arrowhead). A high concentration of IGF-IIR immunostain is also seen in blood vessel walls (double arrowheads). Scale bar = 25 μ m in A–C; 50 μ m in D–I.

investigated the spatiotemporal distribution of TNF and its two receptors, TNF-R1 and TNF-R2, in the embryonic SMGs. The two TNF receptors, TNF-R1 and TNF-R2, transduce very different signals (see review, Darnay and Aggarwal, 1997). TNF-R1, known as the "death-receptor," mediates apoptosis but also regulates cytokine gene expression (e.g., IL-6) through the translocation of NF κ B into the nucleus; TNF-R2 only induces differential gene expression and growth through NF κ B translocation (Ashkenazi and Dixit, 1998). TNF and its two receptors are first immunodetected in the *Pseudoglandular Stage*; TNF, TNF-R1, and TNF-R2 are localized throughout the branching epithelia (Fig. 7A–C). In the *Canalicular Stage*, the diffuse distribution of TNF throughout the epithelia persists (Fig. 7D). TNF-R1 is immunodetected in association with the forming lumina; specifically, it can be seen on the ductal epithelial cell surfaces facing the lumina, at sites of initial ductal lumen formation, and in that region of the terminal end buds which will later exhibit apoptosis and lumen formation (Fig. 7E). The pattern of TNF-R2 immunolocalization is similar to that seen for TNF-R1 (compare Fig. 7F to 7E). By the *Terminal Bud Stage*, TNF continues its diffuse terminal end bud and ductal epithelia distribution (Fig. 7G). TNF-R1 and TNF-R2 are detected in ductal and terminal end bud epithelia (Fig. 7H,I). However, the intensity of TNF-R1 immunostain is considerably diminished in epithelia surrounding distinct lumina (compare Fig. 7E to H), suggesting TNF/TNFR1 mediation of the apoptosis seen in lumen formation is diminishing by this stage (see Fig. 3F,G). To evaluate the TNF/NF κ B growth promotion pathway, we investigated the spatiotemporal protein distribution of IL-6, a TNF-responsive cytokine (Katz et al., 1994; Kurokouchi et al., 1998). Since IL-6 elicits a response through the IL-6 receptor (IL-6R), we also analyzed the spatial distribution of IL-6R in developing SMGs. IL-6 and IL-6R are first immunodetected in *Canalicular Stage* epithelia (Fig. 8A,B). By the *Early Terminal Bud Stage*, IL-6 and IL-6R are localized in ductal and terminal bud epithelial cells surrounding the developing lumina (Fig. 8C,D). By *Late Terminal Bud Stage*, IL-6 and IL-6R are colocalized to the basal surfaces of terminal bud epithelia; IL-6R is also present on epithelial surfaces facing ductal lumina (Fig. 8E,F).

TGF- β s and Cognate Receptors

During embryonic SMG morphogenesis, there is almost certainly a regulated balance between growth promotion and growth inhibition. In contrast to the growth promoting function of the EGF-R and IGF-IR pathways, TGF- β s (TGF- β 1, TGF- β 2, and TGF- β 3) have been shown to inhibit cell proliferation (Derynck, 1994; Hu et al., 1998; Hardman et al., 1994; Melnick et al., 1998). The transcripts for all three isoforms were previously identified in embryonic SMGs (Jaskoll et al., 1994b). TGF- β signal transduction requires both TGF- β -RI (type I receptor) and TGF- β -RII (type II receptor; Hu et al., 1998). TGF- β initially binds to TGF- β -RII which then triggers heterodimerization with TGF- β -RI to induce downstream TGF- β signal transduction. In the *Initial Bud Stage*, TGF- β 2 and TGF- β 3 are immunolocalized throughout both epithelium and mesenchyme (Fig. 9A,B); TGF- β 1's immunolocalization is similar to that seen for TGF- β 2 and TGF- β 3 proteins (data not shown). By contrast, TGF- β -RII (Fig. 9C) and TGF- β -RI (data not shown) are primarily seen in the SMG epithelium and, to a much lesser extent,

in surrounding mesenchyme. By the *Pseudoglandular Stage*, TGF- β 1, TGF- β 2, TGF- β -RI, and TGF- β -RII are immunolocalized in the branching epithelia whereas TGF- β 3 retains its epithelial and mesenchymal distribution (Fig. 9D–H).

By the *Late Canalicular Stage*, different cell-specific patterns for TGF- β isoforms are seen (Fig. 10). TGF- β 1 and TGF- β 2 retain their epithelial distribution (Fig. 10A,B); by contrast, TGF- β 3 is immunolocalized throughout the mesenchyme and extracellular matrix but is absent from the epithelia (Fig. 10C). TGF- β -RII, the receptor which binds TGF- β s, is immunolocalized in ductal and terminal bud epithelial cells surrounding the forming lumina (Fig. 10E); TGF- β -RI exhibits a wider distribution throughout the SMG epithelia (Fig. 10D). By *Early Terminal Bud Stage*, the patterns of TGF- β isoform and TGF- β -RI immunolocalization are similar to that seen in the *Late Canalicular* gland (data not shown). At this stage, TGF- β -RII's spatial distribution has become more restricted, being largely limited to ductal epithelia (Fig. 10F). Shifting patterns of TGF- β ligand and receptor localization during progressive differentiation suggest changing spatial requirements for inhibition of epithelial cell proliferation.

TGF- β 2 and TGF- β 3 Null Mice

Given that TGF- β s have been shown to be an important regulator of embryonic salivary gland morphogenesis *in vitro* and *in vivo* (Hardman et al., 1994; Jaskoll et al., 1994a) and that both TGF- β 2 and TGF- β 3 null mice exhibit abnormal lungs (Kaartinen et al., 1995; Sanford et al., 1997), we postulated that TGF- β 2 and/or TGF- β 3 are essential for normal *in vivo* embryonic salivary gland development. Thus, we evaluated perinatal and neonatal TGF- β 2 and TGF- β 3 null mice. As shown in Figure 11, no detectable SMG differences are evident between TGF- β 2^{-/-} and TGF- β 2^{+/+} or TGF- β 3^{-/-} and TGF- β 3^{+/+} SMGs. This would indicate that normal *in vivo* SMG branching morphogenesis occurs in the absence of one of the functional TGF- β pathways.

DISCUSSION

The mouse neonatal submandibular salivary gland (SMG) is comprised of a network of large and small ducts which terminate in lumen-containing, presumptive acini that express two unique intracellular species of embryonic mucin (Jaskoll et al., 1998). Progressive prenatal morphogenesis begins as a solid outgrowth from the oral epithelium around E11.5 (Fig. 1). To arrive at its newborn anatomy, the SMG must undergo cell growth promotion, cell growth inhibition, and programmed cell death (apoptosis) (Figs. 2,3). Epidermal growth factor (EGF), transforming growth factor- α (TGF- α), insulin-like growth factor-2 (IGF-II), tumor necrosis factor (TNF), interleukin-6 (IL-6), and transforming growth factor- β (TGF- β) make their appearance in this process at specific times and places (Figs. 4–11; Table 1).

In the *Pseudoglandular Stage*, the substantial cell proliferation seen in the solid, budding epithelium is coincident with TGF- α and EGF-R epithelial expression; there is no detectable EGF. At this critical initial stage of development, presence and absence of TGF- α and EGF, respectively, may be related to functional necessity. Derynck (1992) has reported that TGF- α is an order of magnitude more potent than EGF in several proliferation-dependent

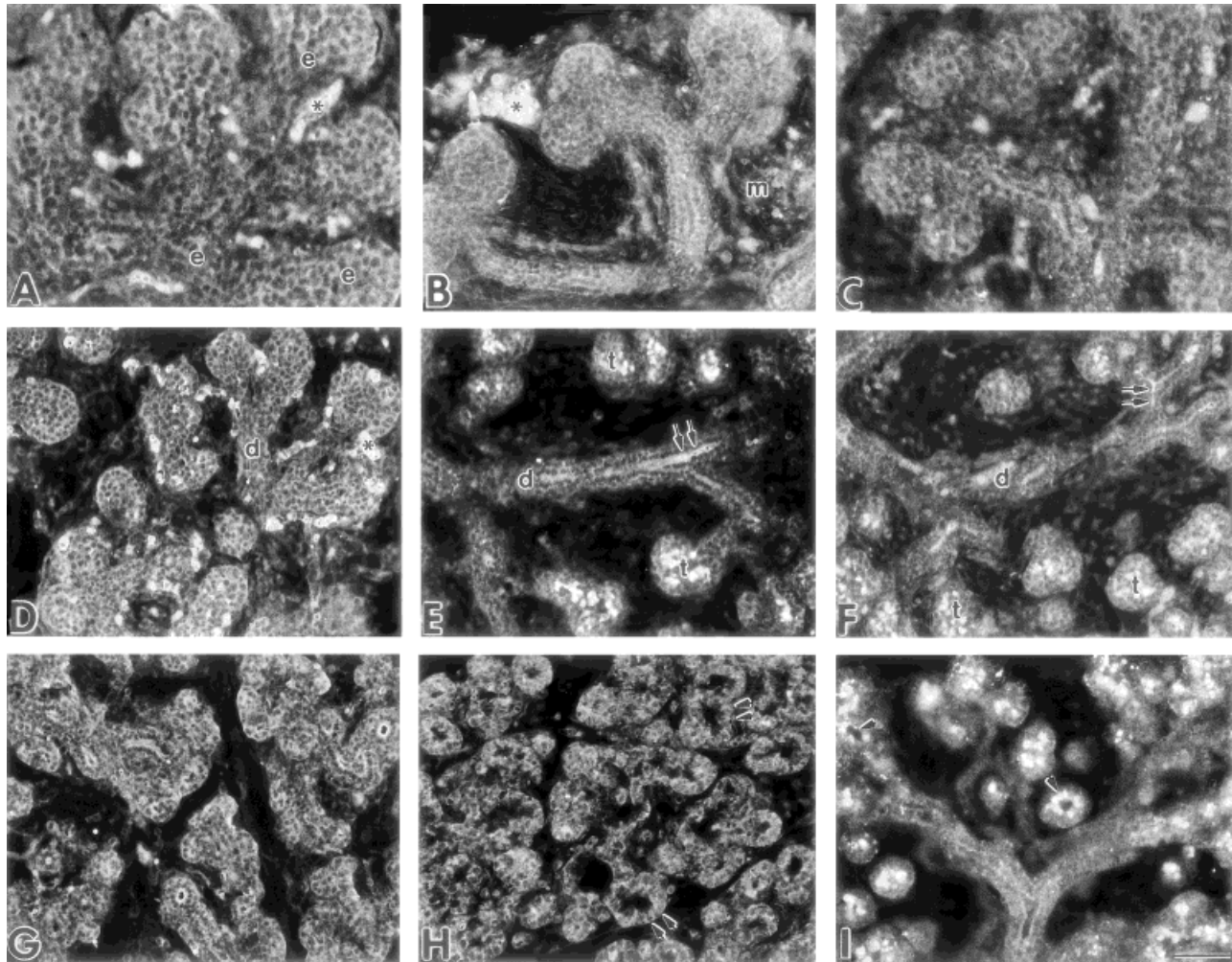


Fig. 7. Immunolocalization of TNF, TNF-R1, and TNF-R2. A–C: *Pseudoglandular Stage*. A: TNF. B: TNF-R1. C: TNF-R2. TNF and its two receptors, TNF-R1 and TNF-R2, are immunolocalized in the branching epithelia (e) and are absent from the mesenchyme (m). In addition, TNF and TNF-R1 are also observed in blood vessels (*). D–F: *Canalicular Stage*. D: TNF. E: TNF-R1. F: TNF-R2. TNF is diffusely distributed throughout ductal (d) and terminal end bud (t) epithelia. TNF-R1 is observed on the lumen-facing ductal epithelial surfaces (double arrows), at the sites of lumen formation, and in the center of terminal end buds. The

pattern of TNF-R2 immunolocalization is similar to that seen for TNF-R1, with its intensity of immunostain being weaker than that of TNF-R1. G–I: *Terminal Bud Stage*. G: TNF. H: TNF-R1. I: TNF-R2. TNF is diffusely distributed on terminal end bud epithelia lining the lumina, as well as on ductal epithelia. There is a substantial decrease in TNF-R1 immunostain on ductal and terminal bud epithelia (double arrowheads) surrounding lumina. TNF-R2 is immunolocalized in the central region of terminal end buds, even in the presence of a lumen (arrow head). Scale bar = 50 μ m.

assay systems, suggesting that TGF- α is a superagonist of EGF-R. IGF-II and IGF-IR are very much more evident in the epithelium than in the mesenchyme; IGF-IIR is more uniformly distributed in both tissues. This is consistent with IGF-II/IGF-IR growth promotion, and with the purported need of a functional IGF-IR for mitogenic signaling by EGF-R (Coppola et al., 1994). TNF, TNF-R1, and TNF-R2 are all localized throughout the epithelium. However, there is no evidence of apoptosis nor is there detectable IL-6, a TNF- α responsive mitogen, or its receptor (IL-6R). Finally, TGF- β 1, TGF- β 2, TGF- β -RI, and TGF- β -RII are localized throughout the epithelium; TGF- β 3 is in both epithelium and mesenchyme. TGF- β s are anchored to IGF-IIR for activation (Dennis and Rifkin, 1991; Gleizes et al., 1997) and the latter is uniformly expressed in SMG tissues at this stage. It is reasonable to assume that the

role of TGF- β s at this stage is to preclude unchecked epithelial proliferation, a sort of "mitostat" if you will. Given the apparent absence of TGF- β receptors in the mesenchyme, it is unclear what role TGF- β 3 is playing there.

The *Canalicular Stage* continues to display substantial epithelial cell proliferation, but it is also characterized by the formation of ductal lumina and a significant change in the expression and location of growth factors and cytokines. Many apoptotic nuclei are located at the site of ductal lumina, and a few are even seen in terminal end buds at sites of presumed later lumen formation. TNF expression is still seen throughout the epithelia, but TNF-R1 and TNF-R2 are largely localized to ductal epithelial surfaces facing lumina, sites of initial lumen formation, and the more central areas of terminal buds destined

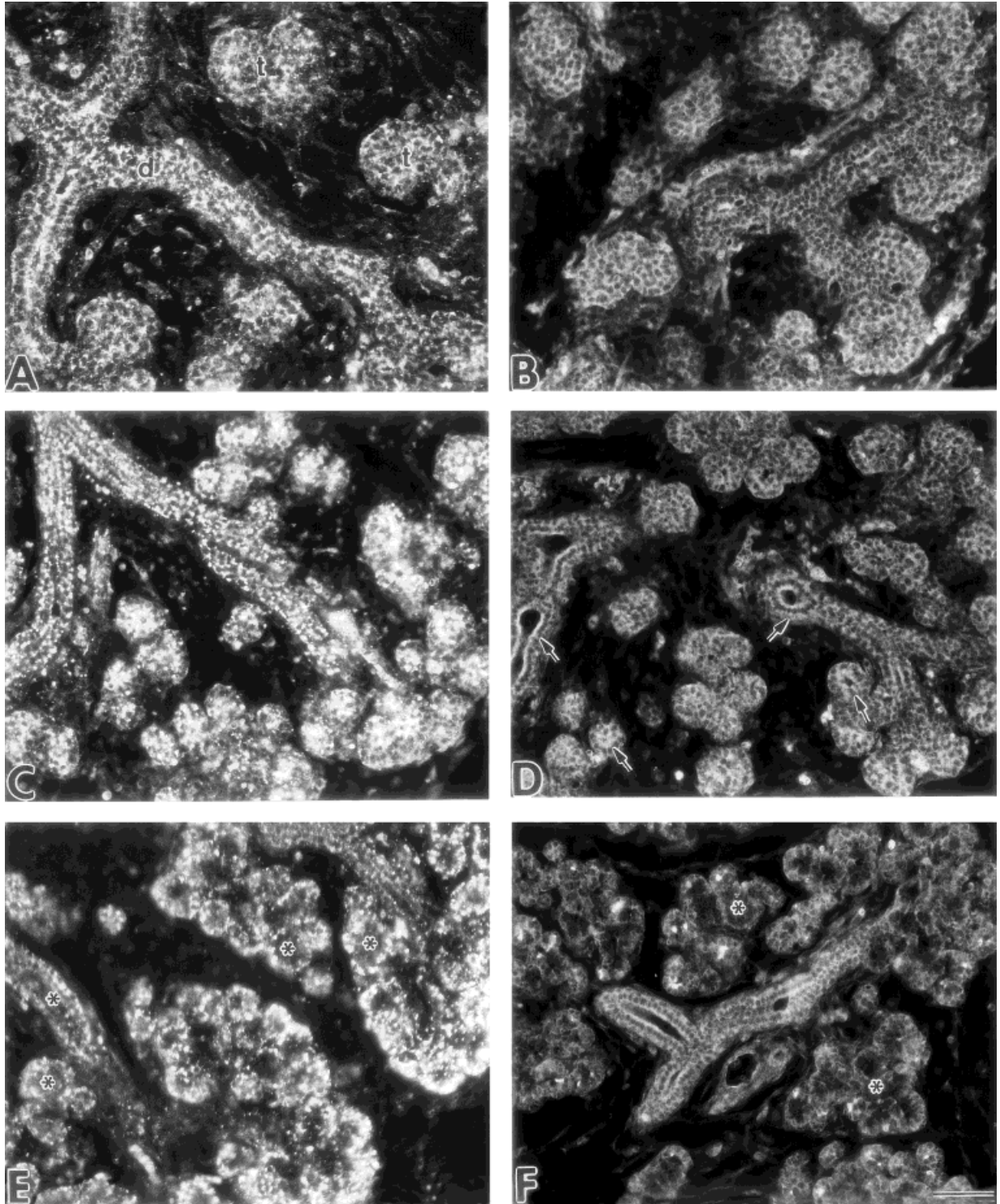


Fig. 8. Immunolocalization of IL-6 and IL-6 receptor (IL-6R). A,B: *Early Canalicular Stage*. C,D: *Early Terminal Bud Stage*. E,F: *Late Terminal Bud Stage*. A,C,E: IL-6. B,D,F: IL-6R. The cytokine IL-6 and its receptor are distributed throughout ductal (d) and terminal end bud (t) epithelia. By the *Early Terminal Bud Stage*, intense IL-6 (C) immunostain is seen in the

center of terminal end buds; IL-6R (D) is immunolocalized on ductal and terminal bud epithelia (arrows). By the *Late Terminal Bud Stage*, IL-6 (E) and IL-6R (F) are colocalized in terminal end bud epithelia, being absent from the lumen-facing epithelial surfaces (*). Scale bar = 50 μ m.

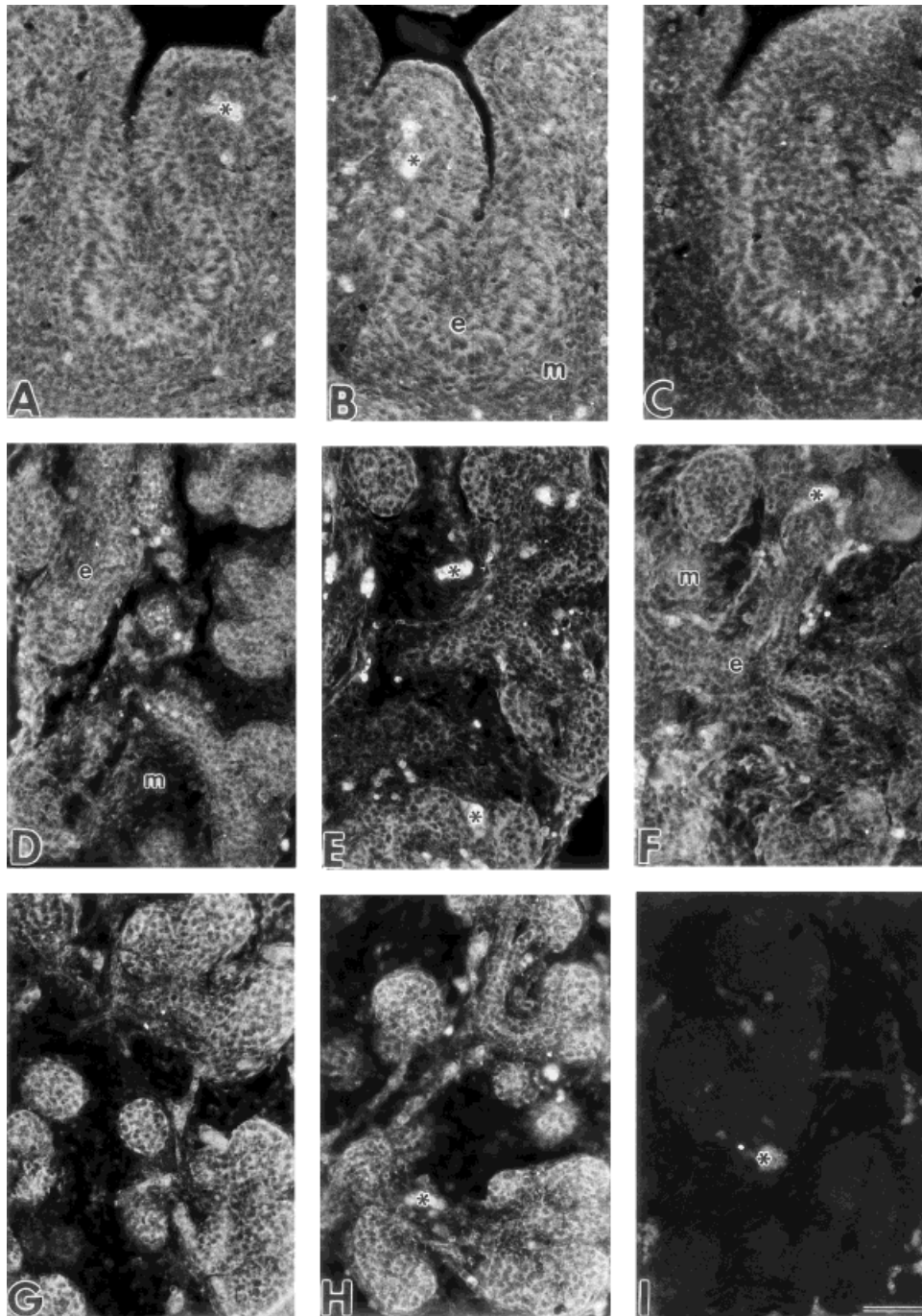


Fig. 9. Immunolocalization of TGF- β s and cognate receptors in the *Initial Bud* and *Pseudoglandular Stages*. A–C: *Initial Bud Stage*. TGF- β 2 (A) and TGF- β 3 (B) are immunolocalized throughout the epithelia (e) and mesenchyme (m). TGF- β -RII (C) and TGF- β -RI (data not shown) are primarily immunodetected in the epithelium (e) and, to a lesser extent, in the adjacent mesenchyme (m). D–I: *Pseudoglandular Stage*. At this stage

of development, TGF- β 1 (D), TGF- β 2 (E), TGF- β -RI (G) and TGF- β -RII (H) are primarily immunodetected throughout the branching epithelia; only TGF- β 3 (F) is distributed throughout epithelia and mesenchyme. The control section (I) is routinely negative. Note that blood cells and blood vessels (*) autofluorescence. Scale bar = 25 μ m in A–C; 50 μ m in D–I.

for apoptosis and lumen formation. IL-6 and IL-6R are now detectable in epithelia. Recall that TNF-R1 is the apoptosis receptor and TNF-R2 promotes mitogenesis via the up-regulation of cytokines such as IL-6 (Ashkenazi and Dixit, 1998). Thus, the TNF/TNF-R signal transduction

pathway may be aiding ductal lumina creation by both promoting death and proliferation in respective neighboring cells. Other factors are also changing appearance and location coincident with changing SMG morphology. EGF is now detected, first in the center of terminal end buds

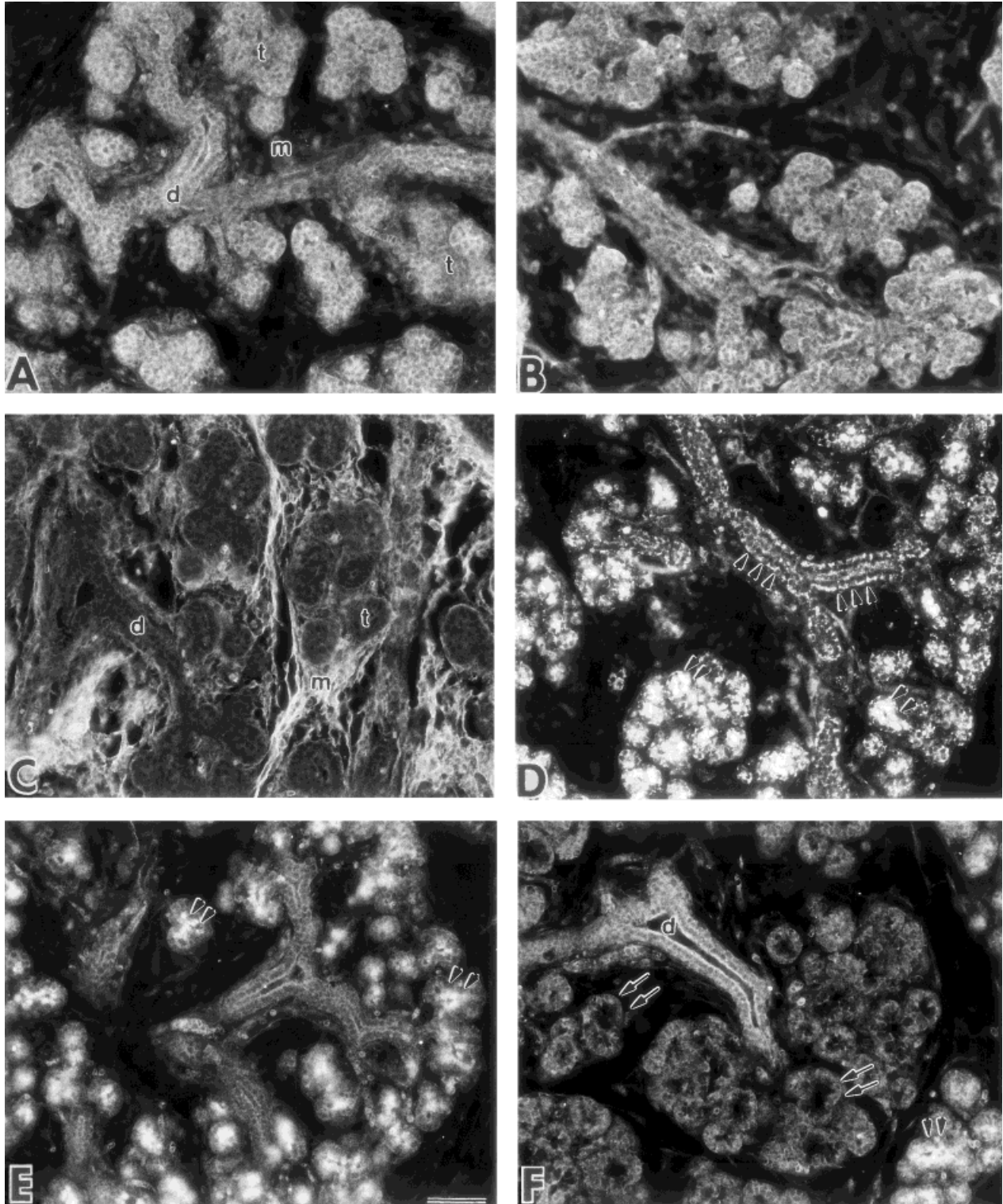


Fig. 10. A-E: Immunolocalization of TGF- β s, TGF- β -RI, and TGF- β -RII in the *Canalicular Stage*. TGF- β 1 (A) and TGF- β 2 (B) retain their distribution in ductal (d) and terminal end bud (t) epithelium. By contrast, TGF- β 3 (C), absent from the epithelia, is immunolocalized throughout the mesenchyme (m) and extracellular matrix. TGF- β -RI (D) is localized on outer ductal epithelia (triple arrowheads), as well as in terminal end bud epithelia (double arrowheads). TGF- β -RII (E) is seen on ductal and

terminal end bud (arrowheads) epithelial cells facing the lumina. F: Immunolocalization of TGF- β -RII in *Terminal Bud Stage*. With development and lumen formation, TGF- β -RII is relatively absent from terminal end bud epithelia (double arrows) with distinct lumina. At this stage, TGF- β -RII is primarily immunodetected on ductal epithelia. Scale bar = 50 μ m.

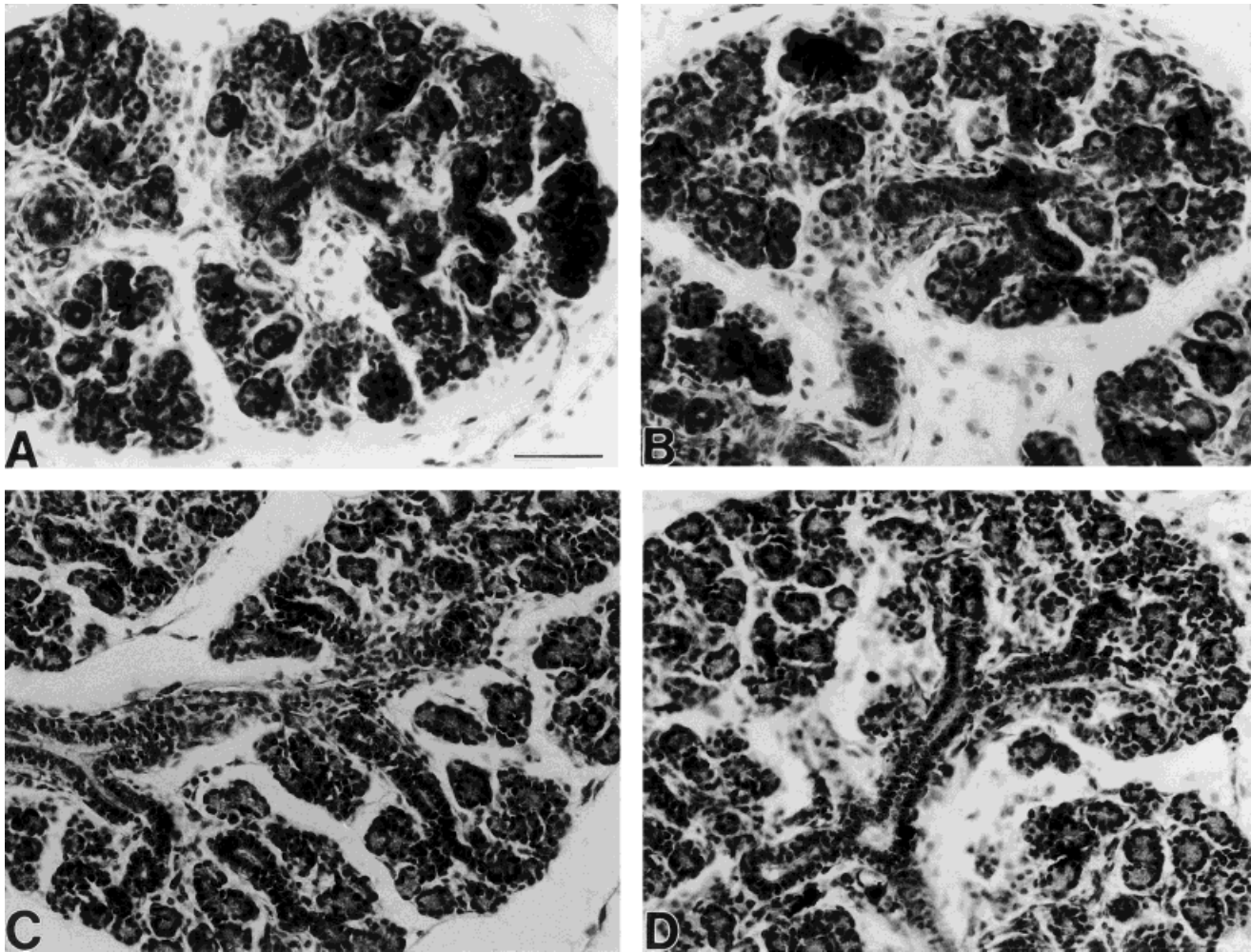


Fig. 11. Salivary gland morphology in TGF- β 2 and TGF- β 3 null mice. **A:** E18.5 TGF- β 2 $^{+/+}$ SMG. **B:** E18.5 TGF- β 2 $^{-/-}$ SMG. **C:** Newborn TGF- β 3 $^{+/+}$ SMG. **D:** Newborn TGF- β 3 $^{-/-}$ SMG. TGF- β 2 and TGF- β 3 null mouse SMGs appear similar to the wildtype glands. Scale bar = 50 μ m.

and later in ductal cells surrounding lumena; TGF- α also localizes to ductal epithelial surfaces facing lumena. EGF-R continues to be present throughout the epithelium. IGF-IR localizes largely to ductal epithelia as well, perhaps only to assist EGF-R signalling as noted above, for IGF-II is now located elsewhere—on the periphery of terminal end buds and, in lesser amounts, in the mesenchyme. IGF-IIR is in the mesenchyme and will serve to sequester the IGF-II located there. Finally, TGF- β 1 and TGF- β 2 are localized throughout the epithelia and TGF- β 3 is now in the mesenchyme only. While TGF- β -RI is also distributed throughout the epithelia, TGF- β -RII is located in ductal and terminal bud epithelia adjacent to present or future lumena. Since TGF- β signal transduction requires both receptors (Hu et al., 1998), it appears that the mitostatic function is reserved for lumena-facing epithelia and presumptive sites of lumena formation. Thus, we see in the *Canalicular Stage* that most of the presumed mitogenic, mitostatic, and apoptotic functions are centered around sites of future ducts and proacini.

In the *Terminal Bud Stage*, cell proliferation begins to diminish, mostly confined to terminal end bud epithelia;

numerous apoptotic nuclei are seen in areas of forming lumena, but little elsewhere. Growth factor and cytokine localization remain concomitant, if more pronounced, with lumena formation in the patterns noted in the *Canalicular Stage*. Coincident with few apoptotic nuclei seen around large distinct lumena, the apoptotic receptor, TNF-R1, is barely present as well. Interestingly, IL-6 and IL-6R colocalize to terminal end bud epithelia not facing lumena. Of particular interest, it is at this stage that two unique embryonic mucin protein isoforms make their appearance within terminal end bud epithelia, especially that associated with forming lumena (Jaskoll et al., 1998).

For the mitogenic, mitostatic, and apoptotic processes that serve SMG morphodifferentiation, the functional scenarios regarding EGF, TGF- α , IGF-II, TNF, and IL-6 clearly are in no way mutually exclusive, but rather cooperative and to some extent redundant. For example, TGF- β 2 and TGF- β 3 null mice develop normal SMGs (Fig. 11) as do TGF- β 1 null mice (Shull et al., 1992; Kulkarni et al., 1993). This may be due to rescue of targeted gene disruption in the embryo by maternal sources of TGF- β (Letterio et al., 1994). It may also be that one isoform can

TABLE 1. Embryonic SMG stage-specific expression of TGF- α /EGF, IGF, TGF- β , TNF, and IL-6 signaling molecules

Stage	Epithelium	Mesenchyme
Initial bud	TGF- α , EGF-R, IGF-II, IGF-IR, IGF-IIR, TGF- β 1, 2, 3; TGF- β -RI, TGF- β -RII	TGF- α , EGF-R, IGF-II, IGF-IR, IGF-IIR, TGF- β 1, 2, 3; TGF- β -RI, TGF- β -RII
Pseudoglandular	TGF- α , EGF-R, IGF-II, IGF-IR, IGF-IIR, TNF, TNF-R1, TNF-R2 TGF- β 1, 2, 3; TGF- β -RI, TGF- β -RII	IGF-II, IGF-IIR, TGF- β 3
Canalicular	TGF- α , EGF, EGF-R, IGF-II, IGF-IR, IGF-IIR, TNF, TNF-R1, TNF-R2, IL-6, IL-6R TGF- β 1, 2; TGF- β -RI, TGF- β -RII	IGF-II, IGF-IIR, TGF- β 3
Terminal bud	TGF- α , EGF, EGF-R, IGF-II, IGF-IR, IGF-IIR, TNF, TNF-R1, TNF-R2, IL-6, IL-6R TGF- β 1, 2; TGF- β -RI, TGF- β -RII	IGF-II, IGF-IIR, TGF- β 3

compensate for the absence of another or that other mitostatic factors replace lost TGF- β function. On the other hand, EGF-R null mice have a dramatic decline (though not an absence) of branching morphogenesis (Fig. 5), indicating that the TGF- α /EGF/EGF-R pathway is critical to normal branching number (euplasia) but not to branching *per se*. This supports the *in vitro* studies of Nakanishi et al. (1987) which show that branching can occur in the absence of epithelial growth, and that of Kashimata and Gresik (1997) which demonstrates a dose-dependent inhibiting effect of branching using a tyrosinase inhibitor of EGF-R function.

Finally, the functional proteins reported here are by no means an exclusive set. For example, corticosteroid appears to modulate branching morphogenesis by down-regulating TGF- β s, particularly TGF- β 2 (Jaskoll et al., 1994b). Endogenous levels of SMG corticosterone increase by more than 50-fold from E14 to E18, and its receptor (GR) by more than 11-fold. Other growth factors localized in embryonic salivary glands include platelet-derived growth factor A (PDGF-A) and its receptor (Orr-Urtreger and Lonai, 1992), scatter factor/hepatocyte growth factor (SF/HGF) and its receptor (Sonneberg et al., 1993), and activin, follistatin, and activin receptor (Ritvos et al., 1995). Molecularly, salivary gland morphogenesis is obviously complex. We believe, however, that if one devises first descriptive and then functional studies of multiple, related signaling molecules within the context of stage-specific cell division, apoptosis, and mucin protein expression, it need not remain obscure.

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