Mouse Submandibular Gland Morphogenesis: a Paradigm for Embryonic Signal Processing

Michael Molnick and Tina Jaskoll

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Mouse Submandibular Gland Morphogenesis: A Paradigm for Embryonic Signal Processing

Michael Melnick*
Tina Jaskoll
Laboratory for Developmental Genetics, DEN-4266, MC-0641, University of Southern California, 925 W. 34th Street, Los Angeles, CA 90089-0641, USA; *corresponding author, mmelnick@hsc.usc.edu

ABSTRACT. Signal processing is the *sine qua non* of embryogenesis. At its core, any single signal transduction pathway may be understood as classic Information Theory, adapted as an open system such that, because of networking, the "receiver" is presented with more information than was initially signaled by the "source". Over 40 years ago, Waddington presented his "Epigenetic Landscape" as a metaphor for the hierarchical nature of embryogenesis. Mathematically, Waddington's landscape may be modeled as a neural net. The "black box" of the neural net is an interacting network of signal transduction pathways (using hormones, growth factors, cytokines, neurotransmitters, and others) which inform the Boolean logic gates. An emerging theme in developmental biology is that defined sets of epigenetic circuits are used in multiple places, at multiple times, for similar and sometimes different purposes during organogenesis. As we show here, submandibular gland embryonic and fetal development is a splendid paradigm of these epigenetic circuits and their phenotypic outcomes, such as branching and lumen formation.

Key words. Embryonic, submandibular gland, signal transduction.

(I) *Introduction*

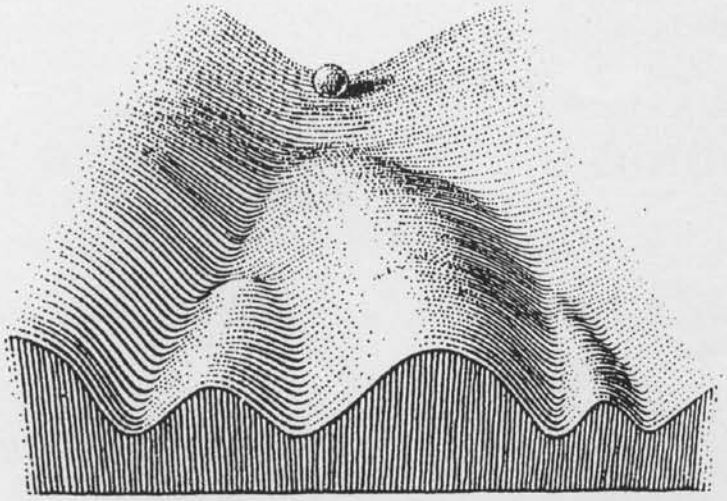
With great flare for the dramatic, the 2nd century Greco-Roman physician, Galen (Claudius Galenus), made a number of prescient observations about embryogenesis in his book **On The Natural Faculties**:

> "For that which was previously semen [known now as the fertilized egg], when it begins to procreate and to shape the animal, becomes, so to say, a special *nature*. For in the same way that Phidias possessed the faculties of his art even before touching his material, and then activated these in connection with this material...so it is with the semen: its faculties it possessed from the beginning, while its activities it does not receive from its material, but it manifests them in connection therewith." (p. 131)
>
> "[Erasistratus] imagines that animals grow like webs, ropes, sacks, or baskets, each of which has, woven on its end or margin, other material similar to that of which it was originally composed. But this most sapient sir, is not growth, but genesis!...Growth belongs to that which has already been completed in respect to its form, whereas the process by which that which is still *becoming* attains its form is termed not growth but genesis. That which *is*, grows, while that which *is not*, becomes." (pp. 137-139)

Thus were planted the seeds of modern developmental biology.

Nearly 1800 years later, with the benefit of more than 100 years of masterful experimentation in embryology and genetics, C.H. Waddington (1957) presented his "Epigenetic Landscape" (Fig. 1) as a metaphor for the hierarchical nature of embryogenesis, an antidote, if you will, to a kind of genetic determinism which seeks to reduce emergent developmental phenomena to nucleotide sequences (*cf.* Strohman, 1997). Mathematically, Waddington's landscape may be modeled as a cellular neural network which uses binary bits (0,1) for data storage and processing and Boolean logic for program execution (if x is 1 AND y is 0 AND z is 1). The underlying jumble of guy wires, giving shape to the canopy, is the "black box", an interacting network of signal transduction pathways (using hormones, growth factors, cytokines, neurotransmitters, and others) which inform the Boolean logic gates (AND, OR, NOT). Even if this is too simplistic, it is quite heuristic. Nonetheless, biologic systems are in fact non-linear, and a more accurate biocybernetic model would include a fuzzy neural network (Kosko, 1992).

It has been mathematically demonstrated that merely 40 genes *could* produce entirely specific cell lineages for about one million differentiated states (Gierer, 1973). Certainly this is not a reality. Nevertheless, an emerging theme in developmental biology is that defined sets of epigenetic circuits are used in multiple places, at multiple times, for similar and sometimes different purposes during organogenesis. As we will show



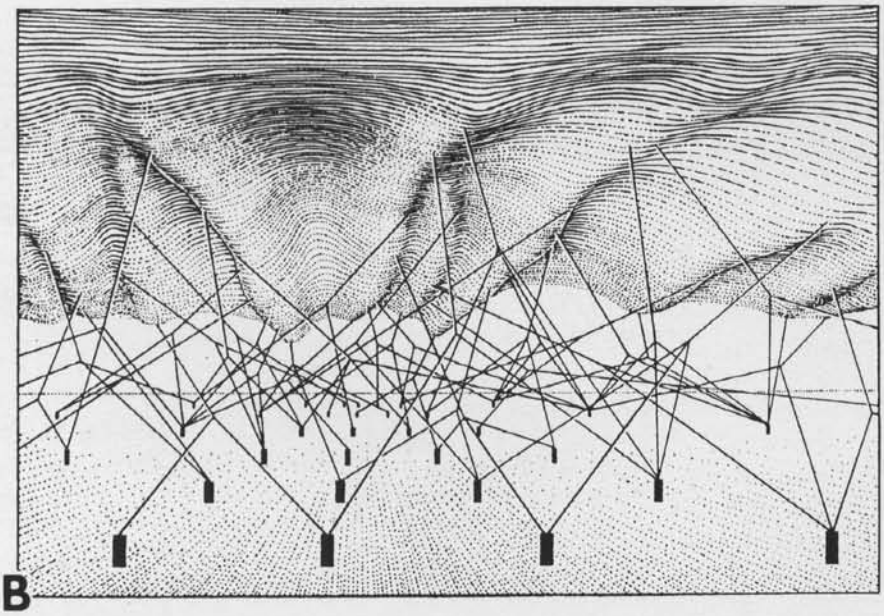


Figure 1. Waddington's "Epigenetic Landscape" (Waddington, 1957). (A) "The path followed by the ball, as it rolls down towards the spectator, corresponds to the developmental history of a particular [organ]. There is first an alternative, towards the right or the left. Along the former path, a second alternative is offered; along the path to the left, the main channel continues leftwards, but there is an alternative path which, however, can only be reached over a threshold." (B) Interacting network of signal transduction pathways. "The pegs in the ground represent genes; the strings leading from them the [pathways initiated by gene expression]. The modeling of the epigenetic landscape, which slopes down from above one's head towards the distance, is controlled by the pull of these numerous guy-robes [pathways] which are ultimately anchored to the genes."

here, submandibular gland (SMG) embryonic and fetal development is a paradigmatic model of these epigenetic circuits and their phenotypic outcomes.

(II) *Branching Morphogenesis of* SMG

Branching morphogenesis is a fundamental embryologic process in many developing organs: salivary gland,

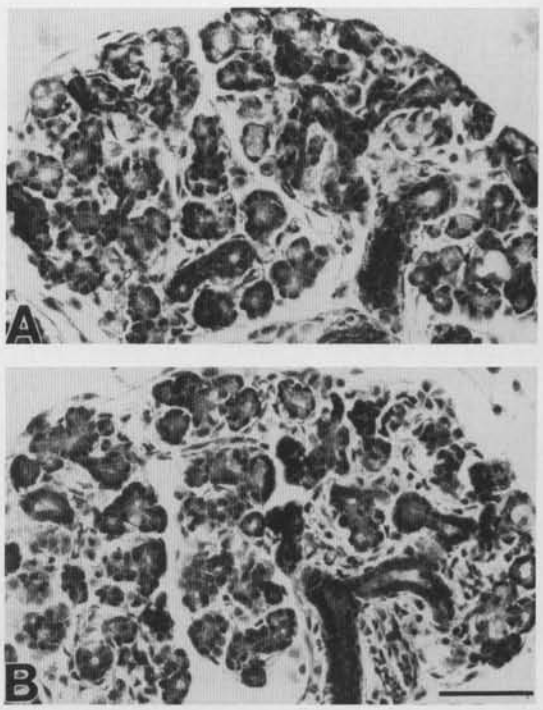


Figure 2. Msx-2 null mouse SMG. (A) Newborn Msx2+/+ SMG. (B) Newborn Msx2-/- SMG. Msx2 null mouse SMG morphology is similar to that seen in the wild-type mouse. Bar = 50 μm.

mammary gland, lung, pancreas, and kidney (see review, Wessells, 1977). Branching organs achieve tree-like (*e.g.*, lung) or bush-like (*e.g.*, SMG) morphology through a program of repetitive, self-similar furcations that serve as branch points for new epithelial outgrowths (Mandelbrot, 1983; Spooner *et al.*, 1989). SMG branching is dependent on epithelial-mesenchymal interactions (EMI), with the mesenchyme being permissive or instructive (Wessells, 1977; Nakanishi *et al.*, 1987; Cutler and Gremski, 1991). Several recent studies suggest a critical role for Msx-2, a homeobox-containing transcription factor, in these cell-cell inductive interactions (Jowett *et al.*, 1993; Liu *et al.*, 1994; Mina *et al.*, 1995). Msx-2 mRNA is expressed in organs dependent on EMI (*e.g.*, tooth, mandible, limb), and this expression is observed only in the presence of EMI. Our recent studies are consistent with these findings (Melnick and Jaskoll, 1997; Jaskoll *et al.*, 1998a). First, Msx-2 mRNA transcripts and protein are expressed in developing SMG epithelium at sites relevant to EMI. Second, transgenic mice that overexpress the Msx-2 transgene exhibit larger, more branched SMGs compared with their wild-type (control) littermates. Nevertheless, Msx-2 is

not absolutely essential to successful SMG EMIs and branching. Our investigation of Msx-2 null mice obtained from Richard Maas' laboratory reveals normal SMG branching morphogenesis (Fig. 2).

(A) SMG DEVELOPMENTAL STAGING

SMG morphogenesis is best conceptualized in stages. In the *Initial Bud Stage*, proliferation of primitive oral cavity epithelium adjacent to the developing tongue on gestational day 11.5-12 produces a thickened epithelium (Fig. 3A) which grows down into a mesenchymal condensation to form the initial SMG bud (Figs. 3B, 3C). With continued downgrowth and cell proliferation, the SMG primordium becomes a solid, elongated epithelial stalk terminating in a bulb (Fig. 3D). In the *Pseudoglandular Stage*, the solid cord of epithelia elongates and grows by repeated end-bud branching into the surrounding mesenchyme (Fig. 4A). As this stage progresses, the mesenchymal cells become less packed, separated by an expanding extracellular matrix of collagens, fibronectin, and other characteristic proteins (Hardman and Spooner, 1992; Macauley *et al.*, 1997).

By the *Canalicular Stage*, the number of epithelial lobes has increased, the presumptive ducts begin to exhibit distinct lumina lined by cuboidal epithelial cells, and the mesenchyme becomes ever more loosely packed (Fig. 4B). In the *Terminal Bud Stage*, distinct, well-developed lumina are seen in presumptive ducts and terminal end buds (presumptive acini) (Fig. 4C). Later in this stage, lumina are bounded by a single layer of low cuboidal cells with mesenchyme near their basal surfaces (Fig. 4D). The continuity between terminal end-bud lumina and ductal lumina is common but not complete. Within the lumina, one may frequently visualize amorphous material; this material may contain "secretory proteins" previously identified in pre-natal SMGs by Ball and co-workers (Ball *et al.*, 1991; Moreira *et al.*, 1991), as well as debris from apoptotic cells.

Cell proliferation and apoptosis have been investigated (Jaskoll and Melnick, 1999) and are

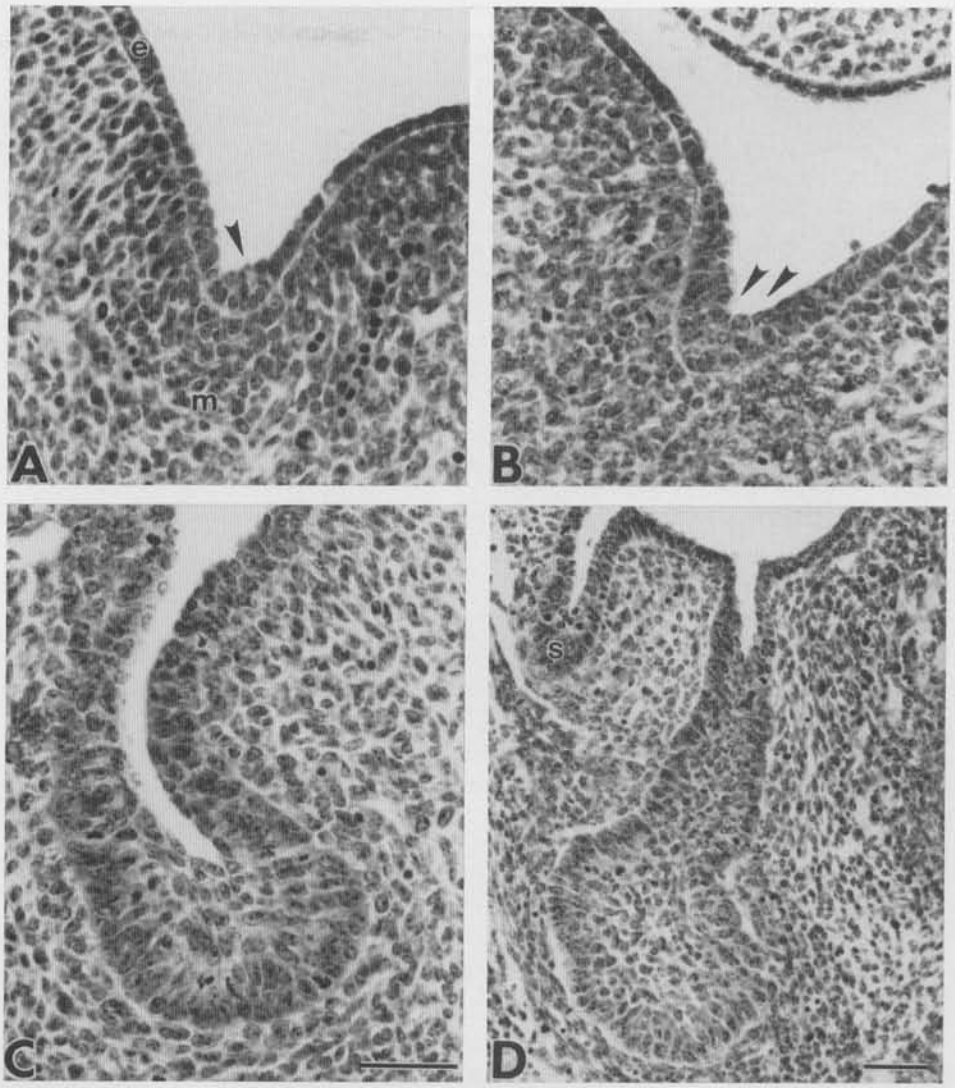


Figure 3. Embryonic SMG *Initial Bud Stage*. (A) The epithelium (e) on the floor of the oral cavity proliferates to form a thickened epithelium (arrow) which grows down into a mesenchymal (m) condensation. (B,C) Continued epithelial thickening (double arrows) and downward growth of the embryonic SMG epithelial bud into the mesenchyme are seen. (D) By the *Late Initial Bud Stage*, the SMG primordium appears as an elongated, solid epithelial stalk with a terminal end bulb. Note that the sublingual gland(s) is beginning to develop. (A-C) Bar = 35 μm; (D) bar = 50 μm.

TABLE 1

SMG Cell Division and Death

	Proliferation		Apoptosis	
Stage	Epithelial	Mesenchymal	Epithelial	Mesenchymal
Initial Bud	+	–	–	–
Pseudoglandular	+	–	–	–
Canalicular	+	–	+	+
Terminal Bud	+	–	+	+

(from Jaskoll and Melnick, 1999)

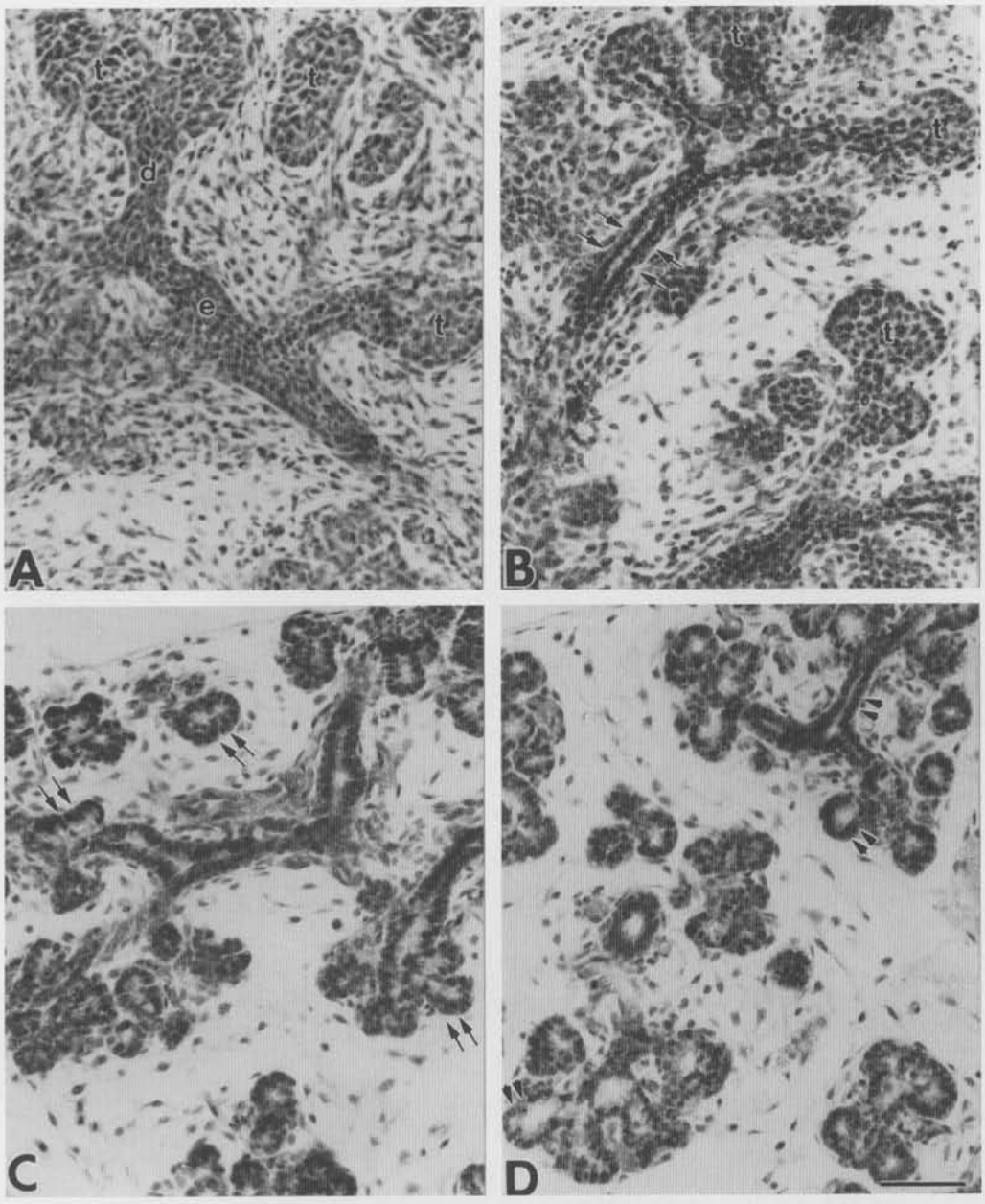


Figure 4. Later stages of embryonic SMG development. (A) In the *Pseudoglandular Stage*, the compact, solid cord of epithelia (e) elongates and branches to form the presumptive ducts (d) and terminal end buds (t). (B) *In the Canalicular Stage*, the presumptive ducts continue to elongate and branch; a substantial increase in the number of terminal end buds (t) is also seen. The ducts begin to exhibit distinct lumena; at sites of lumena, a multilayer of cuboidal cells (arrows) lines the ducts. (C) In the *Early Terminal Bud Stage*, clusters of terminal end buds exhibit distinct lumena surrounded by a multilayer of cuboidal cells (double arrows); the ductal and terminal bud lumena are becoming contiguous. (D) By the Late Terminal Bud Stage, the ductal and larger terminal bud lumena are lined by a single layer of cuboidal epithelial cells (arrowheads). Bar = 50 μm.

summarized by stage in Table 1. There are several observations to note. First, epithelial cell proliferation is found in all stages, even after well-defined lumen formation in the *Terminal Bud Stage*. Second, epithelial apoptosis begins with the onset of lumen formation in the *Canalicular Stage*; it is never seen in the epithelial cells adjacent to mesenchyme and extracellular matrix (see below). Third, as the mesenchyme becomes more loosely packed, apoptosis is seen in these cells as well.

Finally, at birth, the SMG is comprised of a network of large and small ducts which terminate in lumen-containing, presumptive acini that express two unique species of embryonic mucin on their cell surfaces (Jaskoll *et al.*, 1998b).

(B) Embryonic SMG mucin expression

Mucin is the primary histodifferentiation product of submandibular epithelia; it has been extensively characterized in post-natal and adult submandibular glands (Denny *et al.*, 1996, 1997). However, little was known about mucin expression in embryonic submandibular glands. Recently, in our laboratory, embryonic submandibular glands were analyzed and compared with neonatal and adult glands by Northern blot analysis, RNase protection assay, *in situ* hybridization, Western blot analysis, and immunohistochemistry (Jaskoll *et al.*, 1998b). Mucin transcripts are localized to the branching epithelia in the *Pseudoglandular Stage* and older SMGs, with an increase in the hybridization signal being seen in late-embryonic terminal bud and neonatal pro-acinar epithelial cells; a significant 26% increase in transcript level is detected between *Pseudoglandular* and late *Terminal Bud* stages. By contrast, mucin protein is not immunodetected until the *Terminal Bud Stage*; mucin protein is primarily immunolocalized to embryonic terminal bud and neonatal pro-acinar epithelial cell membranes (Fig. 5).

Exogenous corticosteroid (CORT) exposure *in utero* has been shown to enhance embryonic SMG branching morphogenesis (Jaskoll *et al.*, 1994). Exogenous CORT exposure *in utero* also induces

enhanced SMG mucin mRNA and protein expression (Fig. 5). The CORT-stimulated level of mRNA expression and pattern of protein distribution is similar to that seen on the next gestational day in controls. Further, *in vitro* experiments using serumless, chemically defined media demonstrate that CORT is essential for proper embryonic mucin expression (Fig. 6).

Northern blot and Western blot analyses were conducted to determine possible age-specific qualitative differences in SMG mucin transcripts and protein, respectively. Embryos (E17) and neonates (1 day) exhibit two unique mucin transcripts (1.20 kb and 0.85 kb) which are approximately 19% greater or smaller in size than the single (1.01 kb) adult transcript; two embryonic protein isoforms (M_r ~ 110 kDa and 152 kDa) are immunodetected compared with a single adult protein (M_r ~ 136 kDa), with the larger (~ 152 kDa) embryonic isoform persisting in neonatal glands. These results indicate that embryo-specific SMG mucin transcript and protein are expressed by embryonic and neonatal submandibular gland epithelia prior to overt histodifferentiation of SMG acini.

Finally, the precise function of embryonic SMG mucin is unknown. Given that: (1) salivary gland morphogenesis has conclusively been shown to be regulated by epithelial-mesenchymal interactions (see review, Cutler and Gremski, 1991); (2) embryonic mucin protein is immunolocalized to epithelial cell membranes (Jaskoll *et al.*, 1998b); and (3) substantial qualitative differences exist between embryonic and adult SMG mucin mRNA and protein (Jaskoll *et al.*, 1998b), we postulate that embryonic SMG mucin plays a morphogenetic role during cell-cell interactions. Supporting evidence is provided by two studies which show that membrane-bound proteins with a mucin-like domain play functional roles in cell-cell adhesion and interactions (Lasky *et al.*, 1992; Lin *et al.*, 1997). Specifically, the proto-oncogene *Cmyb*, which encodes a transmembrane DNA-binding protein with a mucin-like domain, is required for protein interactions with other cell-surface proteins during mouse fetal liver erythropoiesis and myelopoiesis (Lin *et al.*, 1997). Further, a membrane-bound mucin-like glycoprotein (GlyCAM) has been shown to be the specific receptor which interacts with and binds extracellular L-selectin during lymphocyte differentiation (Lasky *et al.*, 1992). The high-affinity binding of L-selectin is probably due to the extensive glycosylation modifications of GlyCAM and the extracellular presentation of the carbohydrates in the mucin-like structure. Functional studies will sort out the precise *embryonic* role for SMG mucin.

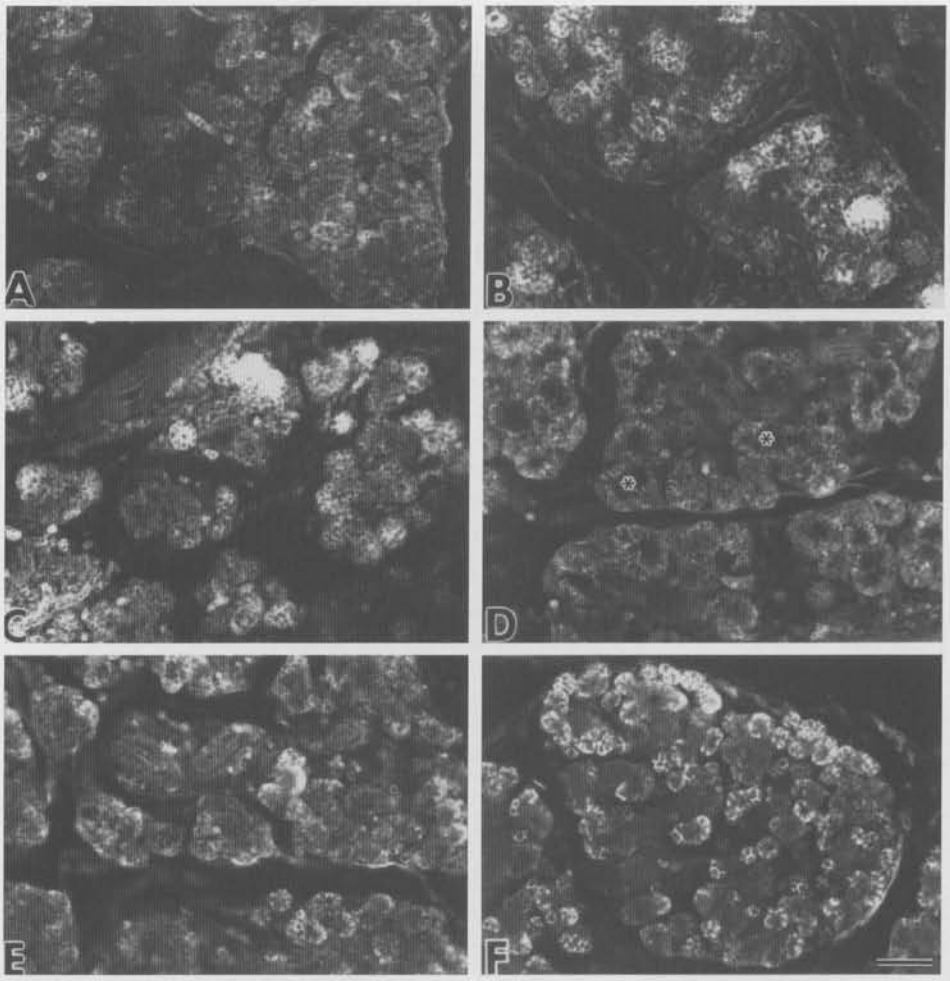


Figure 5. Spatiotemporal distribution of mucin protein in embryonic and newborn SMGs. (A) E17 SMG. (B) E17 CORT-treated SMG. (C) E18 SMG. (D) E18 CORT-treated SMG. (E) E19 SMG. (F) Newborn SMG. Mucin protein is primarily immunolocalized in the cell membranes of terminal bud and pro-acinar epithelial cells. There is a marked increase in immunodetectable mucin protein with gestational age. In the neonate (F), mucin protein exhibits a more restricted immunolocalization in pro-acinar cells. Exogenous CORT treatment substantially increases immunodetectable mucin protein. The CORT-induced pattern of immunolocalization and intensity of immunostain is similar to that seen on the next gestational day in sham-injected controls [*i.e.*, E17 CORT (B) ≈ E18 (C), E18 CORT (D) ≈ E19 (E)]. CORT induction of SMG morphogenesis also results in a substantial increase in lumena size (*). Bar = 50 μm.

(III) *Signal Transduction Pathways*

Communication within and between cell populations during development is typically mediated by hormones,

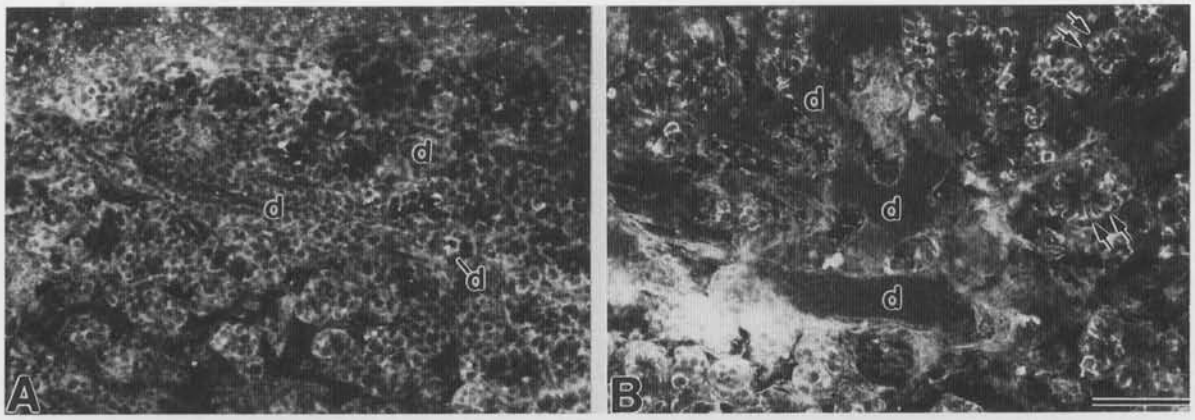


Figure 6. Our *in vitro* experiments suggest that CORT is essential for normal mucin protein expression. With CORT supplementation (B), mucin protein is immunolocalized in terminal end-bud epithelia (arrows) and is absent from ductal (d) epithelia, as it is *in vivo* (Fig. 5). By contrast, control (A) explants exhibit mucin protein in all epithelia, including ductal epithelia. Bar = 50 μm.

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, form, and histodifferentiation. The signal transduction pathways comprise a complex network of mutually dependent organizing mechanisms responsible for progressive differentiation of a multicellular organ such as the SMG.

(A) Growth factors, cytokines, and cognate receptors

The control of cell division is mediated by polypeptide ligands which trigger membrane-associated signal-transducing cascades by binding to their cognate transmembrane receptors (Fig. 7). Ligands binding to receptors with intracellular tyrosine kinase activity include epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF); those binding to receptors with intracellular serine/threonine kinase activity include the transforming growth factor-beta (TGF-β) family. The details of these pathways are beyond the scope of this paper but may be found in Alberts *et al.* (1994) and in links from the Web site of the

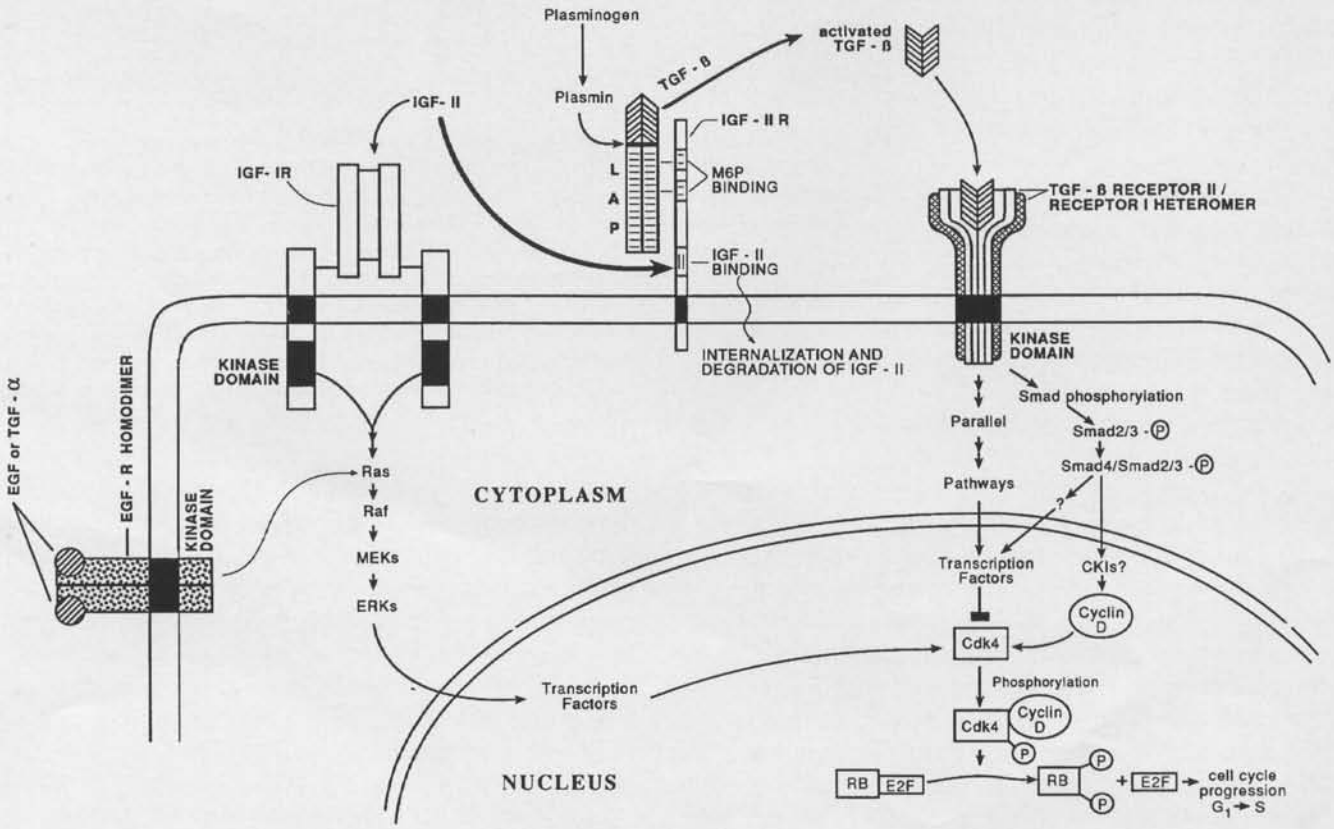


Figure 7. Growth factor signal transduction pathways: EGF (Epidermal Growth Factor), EGF-R (EGF Receptor), IGF-II (Insulin-like Growth Factor II), IGF-R (IGF Receptor), TGF-β (Tranforming Growth Factor β), TGF-βR (TGF-β Receptor). Further details are found in the text.

Society for Developmental Biology (www.sdb.org). Known growth factor, cytokine, and cognate receptor expression in embryonic SMGs are listed in Table 2. EGF, HGF (hepatocyte growth factor), IGF, IL-6 (interleukin 6), and PDGF are mitogenic; the TGF-βs are mitostatic. The tumor necrosis factor (TNF) pathway is multifunctional, both mitogenic (*via* up-regulation of IL-6) and to a lesser extent, apoptotic.

TABLE 2

Expression of Growth Factors, Cytokines, and Their Cognate Receptors in Embryonic SMGs

Growth Factor/Cytokine/Receptor	SMG Stage			
	Initial Bud	Pseudo-glandular	Canalicular	Terminal Bud
EGF (mRNA, protein)	–	–	ep	ep
EGF-R (protein)	ep	ep	ep	ep
HGF (mRNA)		ep/mes	ep/mes	
c-met [HGF receptor] (mRNA)		ep	ep	
IGF-II (protein)	ep/mes	ep	ep/mes	ep/mes
IGF-IR (protein)	ep	ep	ep	ep
IGF-IIR (protein)	ep/mes	ep/mes	ep/mes	ep/mes
IL-6 (protein)	–	–	ep	ep
IL-6R (protein)	–	–	ep	ep
PDGF-A (mRNA)		ep		
PDGF-Rα (mRNA)		mes		
TGF-α (protein)	ep/mes	ep	ep	ep
TGF-β1 (mRNA, protein)	ep/mes	ep	ep	ep
TGF-β2 (mRNA, protein)	ep/mes	ep	ep	ep
TGF-β3 (mRNA, protein)	ep/mes	ep/mes	mes	mes
TGF-β-RI (protein)	ep/mes	ep	ep	ep
TGF-β-RII (protein)	ep/mes	ep	ep	ep
TNF (protein)	–	ep	ep	ep
TNF-R1 (protein)	–	ep	ep	ep
TNF-R2 (protein)	–	ep	ep	ep

Symbols: ep = epithlelium; mes = mesenchyme; – = not present; blank = unknown.
References: EGF/EGF-R (Gresik *et al.*, 1997; Kashimata and Gresik, 1997; Jaskoll and Melnick, 1999).
HGF/c-met (Sonnenberg *et al.*, 1993).
IGF-II/IGF-IR/IGF-IIR (Jaskoll and Melnick, 1999).
IL-6/IL-6R (Jaskoll and Melnick, 1999; Melnick *et al.*, 2000).
PDGF-A/PDGF-Rα (Orr-Urtreger and Lonai, 1992).
TGF-α (Jaskoll and Melnick, 1999).
TGF-βs/TGF-β-Rs (Hardman *et al.*, 1994; Jaskoll *et al.*, 1994; Jaskoll and Melnick, 1999). TNF/TNF-RI/TNF-RII (Jaskoll and Melnick, 1999).

(B) Signaling Networks

It is clear that a wide variety of signaling molecules and their receptors is expressed in the developing SMG. Ligand-receptor binding is the first step in pathways of signal processing that effect specific gene expression and phenotypic change. Typically, signaling pathways are studied as though information processing were linear. However, it is becoming increasingly apparent that pathways interact with one another, and the final biologic response is shaped by this interaction (Bhalla and Iyengar, 1999). This results in signaling *networks* of great complexity and non-linearity.

Signal processing utilizes two primary mechanisms: (1) protein-protein interactions and enzymatic reactions such as protein phosphorylation and dephosphorylation; and (2) protein degradation or production of intracellular messengers (Alberts *et al.*, 1994). Bhalla and Iyengar (1999) have modeled these mechanisms as:

$$A + B \underset{k_b}{\overset{k_f}{\rightleftarrows}} AB$$

$$A + B \underset{k_b}{\overset{k_f}{\rightleftarrows}} C + D$$

where the interactions are completely specified by rate constants (k_b and k_f) and by the initial concentration of each reactant (A, B, C, D), and can be represented by differential equations of the form

$$d[A]/dt = k_b[C][D] - k_f[A][B]$$

They have used this to model the interactions of two EGF-R signaling pathways. The work is elegant, complex, and well worth the effort to understand it. To get a sense of how such networks are operating, we direct your attention to Fig. 7.

SMG branching morphogenesis is characterized by epithelial cell division, and Fig. 7 diagrams a number of important pathways that control growth of the SMG primordia. Experiments with mouse embryo cells *in vitro* indicate that there is an interdependence between receptors IGF-IR and EGF-R; IGF-IR function requires the previous activation of EGF-R, and EGF-R requires a functional IGF-IR (Coppola *et al.*, 1994). The explanation for this may be that both share the Ras signal transduction pathway (Fig. 7).

Some of the other known factors that make this complex network non-linear and adaptive include the following: (1) IGF-IIR binds IGF-II with a very significantly greater affinity than IGF-IR (Jones and Clemmons,

1995); (2) although ligand binding of the IGF-IR is not a *sine qua non* for cell cycle progression, it is probably required for the cell cycle to be maintained at a normal rate (LeRoith *et al.*, 1995); (3) since IGF-II and M6P-bearing molecules (*e.g.*, latent TGF-β) competitively bind to their cognate IGF-IIR sites because of steric hindrance or conformational change, any imbalance in ligand(s) and receptor concentration is likely to alter associated biological functions, such as IGF-II degradation, IGF-II/IGF-IR binding, and TGF-β binding and activation (Vignon and Rochefort, 1992); (4) TGF-β decreases the mRNA expression of both uPA and tPA plasminogen activators and may stimulate PA-inhibitor production (Keski-Oja *et al.*, 1988; Agrawal and Brauer, 1996); (5) plasmin-dependent activation of TGF-β is modulated by surface localization of uPA by its receptor (Odekon *et al.*, 1994); and (6) parallel pathways in TGF-β signaling result in additional "crosstalk" with the EGF, FGF (fibroblast growth factor), and HGF pathways (Engel *et al.*, 1998; Visser and Themmen, 1998). This, then, is a dynamic network that uses a continuous logic to learn its rules from changing conditions (Melnick *et al.*, 1998).

As Bhalla and Iyengar (1999) demonstrate, it is critical that we recognize that these networks exhibit emergent properties such as integration of signals across multiple time scales, generation of distinct outputs depending on input strength and duration, and self-sustaining (autocatalytic) feedback loops. What is meant by "emergent" is that the whole is greater than the sum of its parts. Thus, growth promotion in the SMG *Pseudoglandular Stage* is not located in the property of any single mole-

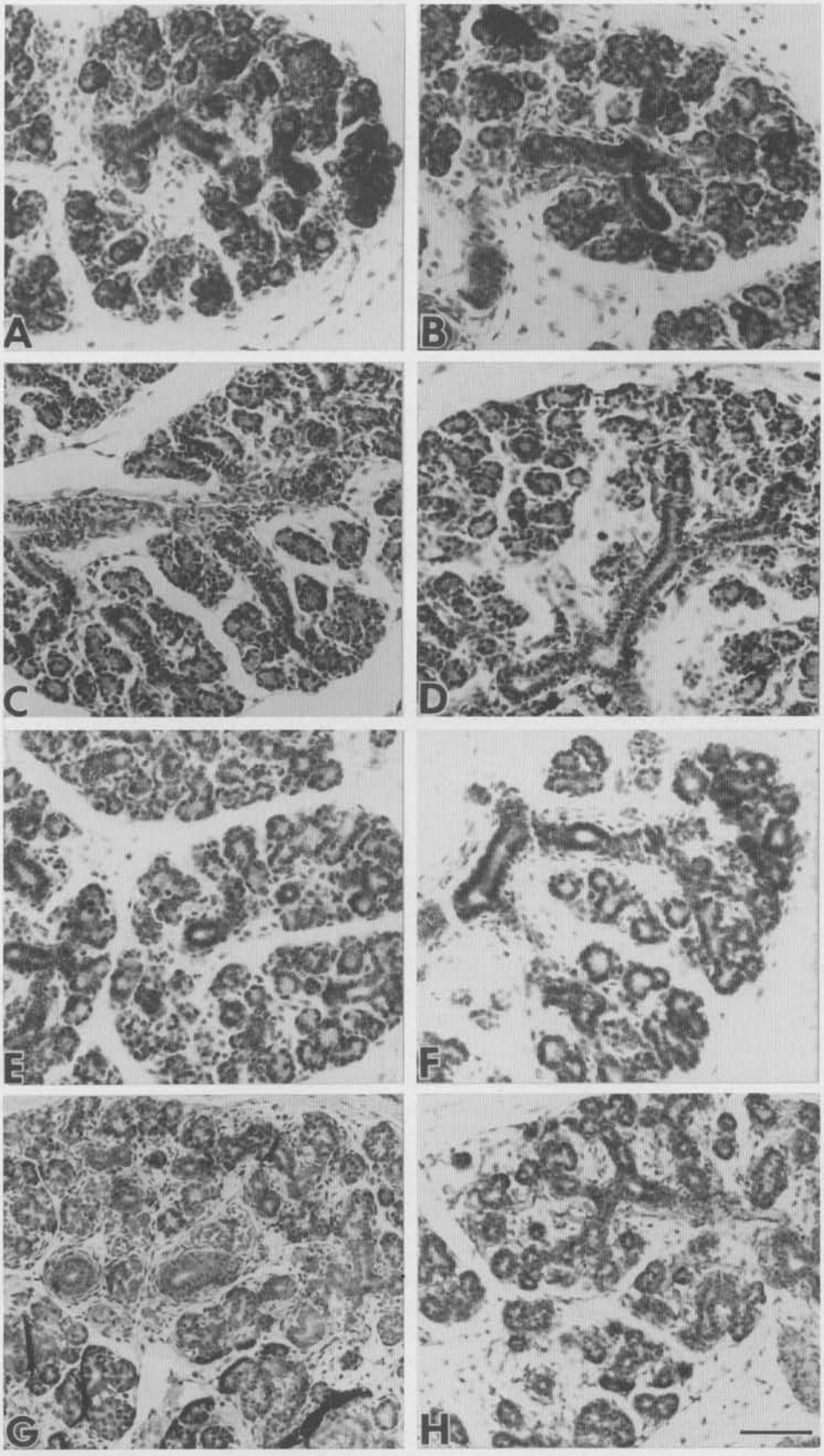


Figure 8. Salivary gland morphology in TGF-β2, TGF-β3, EGF-R, and TACE null mice. (A) E18.5 TGF-β2 +/+ SMG. (B) E18.5 TGF-β2 -/- SMG. (C) Newborn TGF-β3 +/+ SMG. (D) Newborn TGF-β3 -/- SMG. (E) Newborn EGF-R +/+ SMG. (F) Newborn EGF-R -/- SMG. (G) E17.5 TACE +/+ SMGs. (H) E17.5 TACE -/- SMGs. TGF-β2 (B) and TGF-β3 (D) null mouse SMGs are similar to the wild-type glands (A,C). By contrast, EGF-R null mouse SMGs (F) exhibit a substantial decrease in epithelial branch number compared with wild-type glands (E). A decrease in epithelial branch number is also seen in TACE null mouse SMGs (H) compared with wild-type glands (G), although to a lesser degree than seen in EGF-R null mice. Bar = 50 μm.

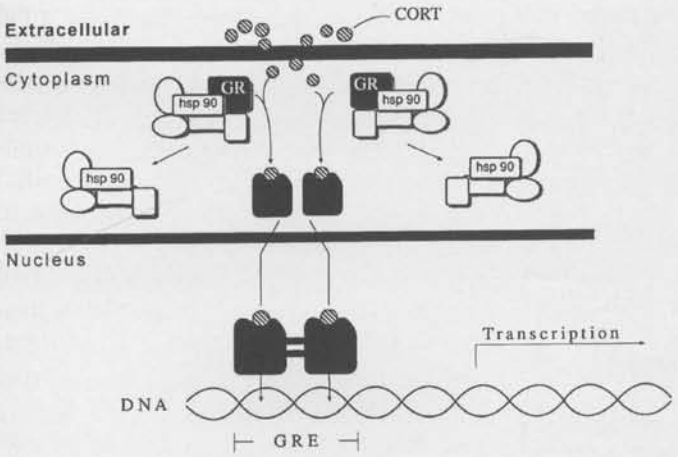


Figure 9. Corticosteroid signal transduction pathway. Lipophilic glucocorticoid (CORT) translocates across the plasma membrane to the cytoplasm and binds to the glucocorticoid receptor (GR). Activation of GR releases the hsp90-dominated complex to which it is bound. CORT/GR translocates to the nucleus, homodimerizes, and binds to a DNA glucocorticoid response element (GRE) in the regulatory region of target genes to up- or down-regulate transcription.

cule but is a collective property of networks of interacting molecules (see Kauffman, 1993, for a detailed explication).

(C) Transgenic clues

There is ample justification to view TGF-βs as important regulators of embryonic SMG morphogenesis *in vitro* and *in vivo* (Hardman *et al.*, 1994; Jaskoll *et al.*, 1994; Jaskoll and Melnick, 1999). However, investigation of perinatal and neonatal TGF-β2 and TGF-β3 null mice (Jaskoll and Melnick, 1999) found no detectable differences between TGF-β2 -/- or TGF-β3 -/- and their wild-type partners (Figs. 8A-8D). This suggests that normal *in vivo* SMG morphogenesis can occur in the absence of functional TGF-β pathways, the developing gland apparently recruiting alternative mitostatic pathways.

Regarding mitogenesis, the EGF-R pathway would appear from *in vitro* and *in vivo* studies to be an important regulator of SMG branching morphogenesis (Kashimata and Gresik, 1997). Investigation of EGF-R null mice (Miettinen *et al.*, 1995) supports this: EGF-R -/- mice have a 50% reduction in terminal buds and a substantial increase in extracellular matrix (Figs. 8E, 8F) as compared with EGF-R +/+ (Jaskoll and Melnick, 1999). Further evidence comes from TACE null mice (Peschon *et al.*, 1998). One of the functions of this metalloproteinase-disintegrin is the proteolytic processing of TGF-α to its active form. Evaluation of TACE null mice SMGs reveals a dramatic reduction in terminal bud branching and an increase of extracellular matrix as compared with their wild-type partners (Figs. 8G, 8H). This is not entirely surprising, since TGF-α is an important EGF-R ligand, particularly in pre-*Canalicular* stages (Table 2). Taken together, these null mice (EGF-R and TACE) provide solid indication that the EGF-R pathway is not essential for SMG initiation, but *is* essential for normal branching number (euplasia). This confirms findings from *in vitro* studies (Kashimata and Gresik, 1997).

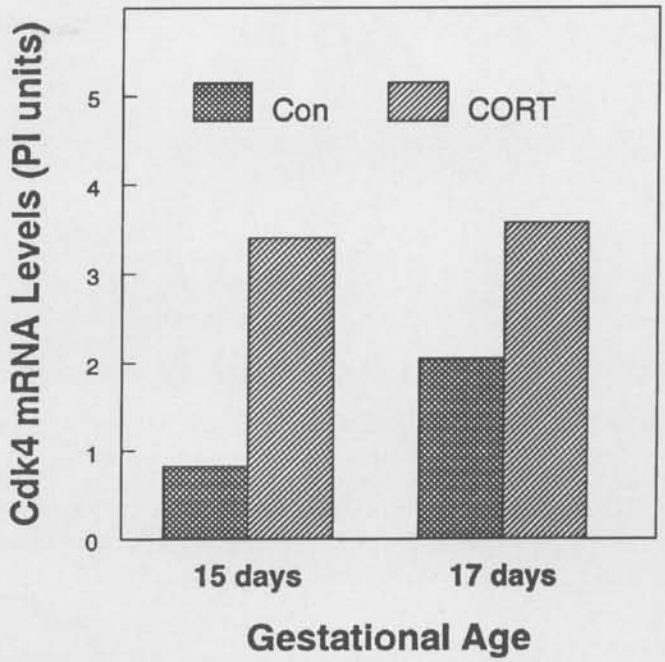


Figure 10. CORT-induced up-regulation of SMG cell proliferation *in vivo* correlates with the level of Cdk4 (cyclin-dependent kinase 4) mRNA transcript; Cdk4 is essential for G_1 S progression through the cell cycle. RNase protection assays demonstrate a 2.5-fold increase in Cdk4 mRNA levels with gestational age (E15 to E17) during normal embryonic development. CORT induces a significant four-fold increase on E15 and a significant 1.75-fold increase on E17 in Cdk4 mRNA levels compared with controls.

(D) Glucocorticoid and SMG branching morphogenesis

Glucocorticoid (CORT) is important to embryonic SMG branching morphogenesis (Jaskoll *et al.*, 1994). Radioimmunoassays first detect CORT in amounts > 2 pg/gland on day 15 of gestation (E15); Western analysis first detects the 96-kDA CORT-receptor (GR) on E14 (0.14 fmol/gland). By E18, SMG CORT has increased more than 50-fold, and SMG GR has increased nearly 11-fold. The SMG GR is functional, as defined by its ability to bind a DNA CORT response element (GRE), on all gestational ages evaluated (14 to 18). Steroid hormone signal transduction (Fig. 9) is different from that of growth factors (Fig. 7). CORT is lipophilic and crosses the cell membrane, where it binds to cytoplasmic GR. Activated ligand-bound receptor is translocated to the nucleus, dimerizes with other ligand-bound receptors, and binds to GREs in the regulatory region of target genes. In essence, the CORT/GR complex serves as a transcription factor, up- and down-regulating gene expression.

Exogenous CORT administration on E12 significantly increases embryonic SMG growth *in utero* and accelerates the rate of SMG morphogenesis by about half a ges-

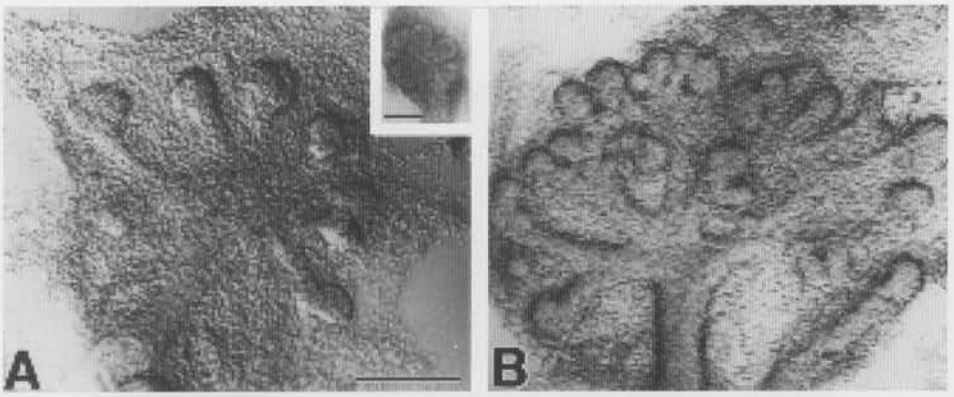


Figure 11. Exogenous CORT supplementation induces SMG branching morphogenesis *in vitro*. E13 SMG primordia, which initially possess 3-5 epithelial lobes (inset), were cultured for 3 days under chemically defined conditions in the absence (A) or presence (B) of CORT supplementation. CORT-treated explants exhibit a significant increase in the number of epithelial lobes compared with control. Bar = 100 μm.

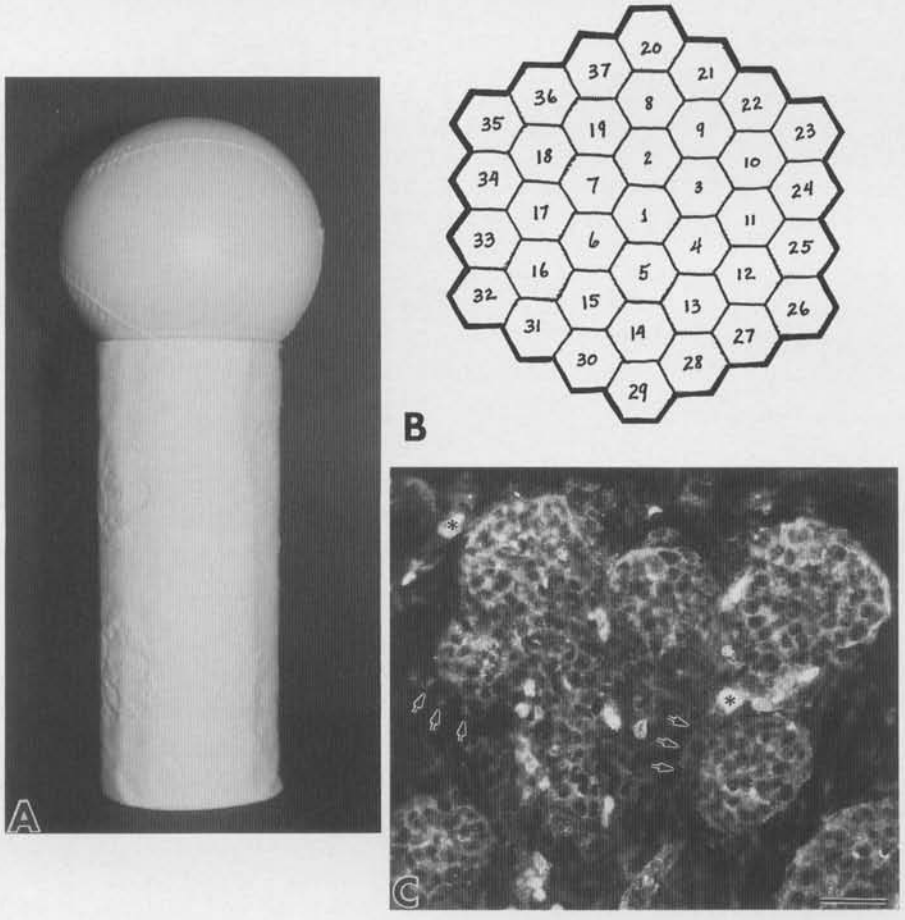


Figure 12. Modeling lumen formation. (A) *The Early Canalicular Stage* branch and end bud may be modeled (stylized) as a solid sphere-cylinder structure, with the seemingly vacant space around it as the extracellular matrix. (B) Cross-sections perpendicular to the long axis of the cylinder and at the widest diameter of the sphere are modeled as a two-dimensional, tessellated, hexagonal array, each element of which has a specific address (1, 2, 3...n). (C) p53 is associated with two functions, cell cycle arrest and apoptosis. In the *Early Canalicular Stage*, p53 protein is diffusely distributed throughout end bud epithelia. However, peripheral epithelia adjacent to extracellular matrix (arrows), and destined to survive, exhibit little immunodetectable p53. p53 is also seen in blood cells (*). Bar = 25 μm.

tational day. This is coincident with a 25-35% decline in TGF-β2 and TGF-β3 expression and almost a four-fold elevation of Cdk4 gene expression by E15 (Fig. 10). Further, E13 SMG primordia (early *Pseudoglandular Stage*), cultured under serumless, chemically defined conditions, demonstrate a significant enhancement of branching morphogenesis when supplemented with CORT (Fig. 11). Without CORT supplementation of these explants, then, SMG branching is significantly delayed. As noted above, CORT is also required for the proper expression of embryonic mucin (Fig. 6).

(IV) *Modeling Lumen Formation*

As we have seen, SMG organogenesis begins as a localized proliferation of the oral epithelium which constitutes the leading edge of an invagination into the underlying mesenchyme. This mass of epithelial cells initially is a single stalk with a single lobule or bud at its distal end. The SMG primordium undergoes branching by repeated furcation at the distal ends of successive epithelial buds. This process produces a bush-like structure at the *Pseudoglandular Stage* comprised of a network of elongated epithelial branches with epithelial buds at their termini. By the *Terminal Bud Stage*, these branches and buds are hollowed out (canalized/cavitated) to form the ductal system and the presumptive acini, respectively. The phenotypic change from pseudoglandular to glandular-like morphology, commencing in the *Canalicular Stage*, is a transition from a three-dimensional mass of cells to a two-dimensional sheet of cells with apical-basal polarity enclosing a lumen. At its simplest, lumen formation may be thought of as the programmed death (apoptosis) of all cells central to the peripheral layer in branches and buds. A first approximation of the histologic and molecular events would suggest, however, that this is a rather complicated process and that it can be profitably modeled.

To be sure, models are, by necessity, highly simplified *thought* experiments. Few have failed to recognize the inherent difficulty of empirical verification, not

least because models attempt to predict one process on the basis of others by reducing Waddington's (1957) "epigenetic landscape" to coefficients in a set of linear equations, a kind of artificial toy world. Nonetheless, we endeavor here to model lumen formation. Although this somewhat charming "game" provides only a rough picture of the canalizing SMG, and may not necessarily be interpreted rigorously, it has certain merits for one who wishes to have some mental picture, however tenuous, for what one is attempting to ponder.

Stylized, the branch and its end bud may be thought of as a sphere-cylinder structure, here modeled as a child's plastic ball atop a toilet-paper tube (Fig. 12A). At first, we imagine this structure as solid with the seemingly vacant space around it as the extracellular matrix (ECM). We must, of course, look inside to visualize what is about to occur. To do this, we take cross-sections perpendicular to the long axis of the cylinder and at the widest diameter of the sphere; the morphology of all cross-sections appears to be identical (Fig. 12B).

We model these cross-sections as a cellular space consisting of a two-dimensional Euclidean space, together with a neighborhood relation defined on this space, *i.e.*, a list of neighbors for each cell (Fig. 12B). Time is marked as discrete "generations" (G = 1, 2, 3, 4). Each cell has a finite list of states. Specific "rules" define the state of a given cell at time $t + 1$ as a function of its own state and the states of its neighbors at time t. von Neumann (1966) calls the list of states, together with the rule governing the state transition of a cell, a transition function. Since cells normally have between five and seven neighbors when packed in two dimensions (Ransom, 1981), it is usually assumed that cells tend, on average, to pack into a hexagonal alignment *in vivo* (Williams and Bjerknes, 1972; Antonelli *et al.*, 1973).

The model presented here (Fig. 12B) is a two-dimensional array comprised of tessellated regular hexagons representing SMG epithelial cells at the start of the *Canalicular Stage*. Each element (cell) has a specific coordinate (1, 2, 3...n). Initially, all cells other than those at the array boundary facing the ECM have six neighbors. Each concentric cell layer is processed consecutively at *each* generation according to a set of rules:

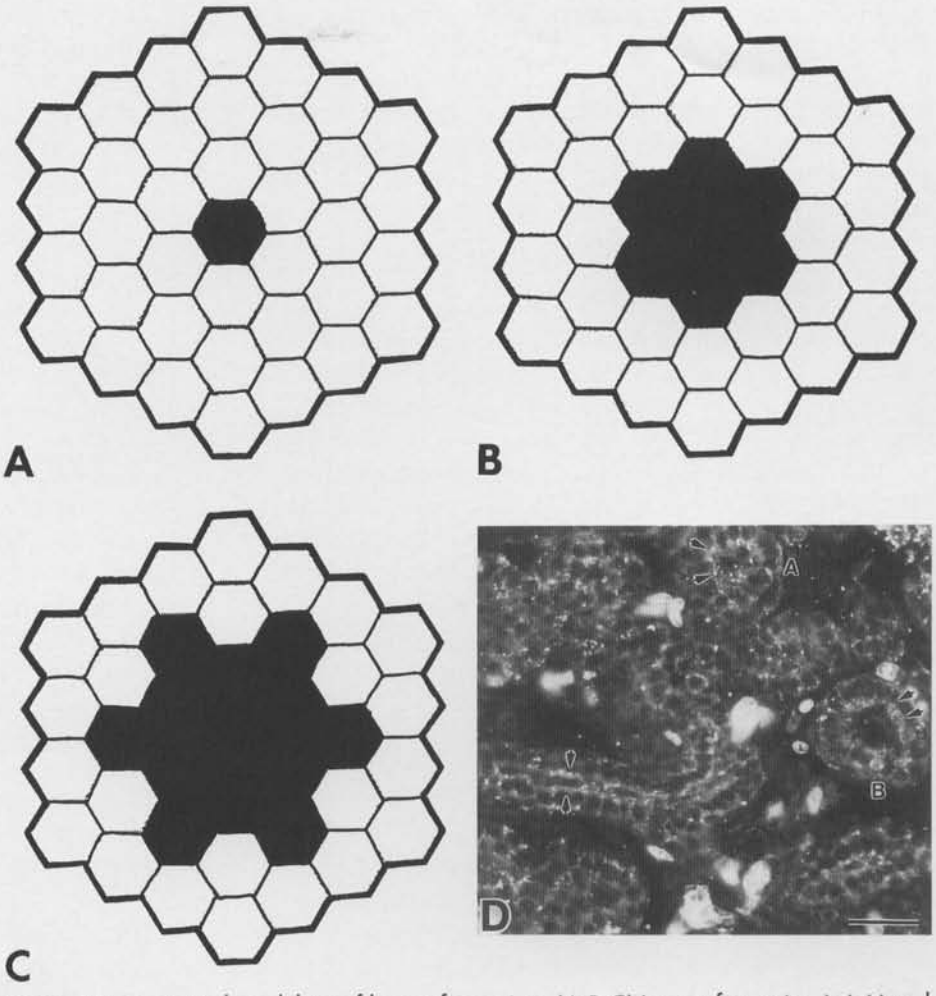


Figure 13. Continued modeling of lumen formation. (A,B,C) Lumen formation is initiated with the death of the central cell (1). Each concentric cell layer is processed consecutively at each generation (t+1, t+2, t+3) according to the set of rules defined in the text. (D) Spatial distribution of p53 protein in *Late Canalicular Stage* SMG. "A" and "B" are the *in vivo* end bud equivalents of hexagonal arrays in 13A and 13B, respectively. p53 immunostaining is most intense in the concentric cell layer next destined for apoptosis (arrowheads). Bar = 25 μm.

(1) Lumen formation is initiated with the death of the central cell (1).
(2) In subsequent generations, each cell with the fewest neighbors dies.
(3) All other cells survive for the next generation.
(4) All cells at the array boundary are privileged—that is, they survive regardless of few neighbors.

Generations 1-4 are represented in Figs. 12 and 13. Generation 5 (not shown) is the *Terminal Bud Stage* with the stabilized luminal phenotype. This is a descriptive or conceptual model which serves as a context into which existing knowledge can be fit to provide an approximation to reality. The rules are informed by our observation of scores of developing SMGs and hundreds of sections (see, *e.g.*, Fig. 13D). Underlying the generation of form are important cognate cellular relations and changes, as well

as gene and protein expression. To appreciate these, we must detail the *initial conditions* of Fig. 12.

The basal surfaces of the array boundary cells are adjacent to that portion of the ECM termed the basement membrane. This fibrillar protein meshwork is composed of laminins, fibronectin, BM-1 proteoglycan, and collagens I, IV, and V (Hardman and Spooner, 1992; Macauley *et al.*, 1997). Epithelial cell attachments to ECM components are mediated by cell-surface receptors called integrins. Integrins are composed of α and β transmembrane subunits selected from among 16α and 8β subunits that heterodimerize to produce more than 20 different receptors which are ligand-specific (Clark and Brugge, 1995). For example, $\alpha 6\beta 1$ integrin binds laminin-1 (Kadoya *et al.*, 1995). Most cells require attachment to the ECM substrate for proper growth, function, and survival (anchorage dependence); the apoptosis resulting from lack of anchorage is termed "anoikis" (Ruoslahti, 1997). Integrin/ECM-ligand anchorage results in chemical and perhaps mechanical (cytoskeletal) signals for survival; loss of ECM anchorage results in an altered cell geometry, an interrupted survival signal, and an apoptosis signal (Chen *et al.*, 1997; Ruoslahti, 1997). Thus, the boundary epithelial cells are a privileged class in that they are programmed for survival from the outset.

The interior cells have no such protection. They are attached only to their neighboring cells by weaker junctions, termed adherens junctions. The core structure of the adherens junction is the cadherin-catenin complex (Kemler, 1993). E-cadherin (epithelial) is a Ca^{2+}-dependent transmembrane cell adhesion glycoprotein; it is anchored in the cytoplasm to actin filament by α and β catenin. Cell-cell attachment is mediated by homophilic interaction between cadherin molecules on adjacent cells (Takeichi, 1991). Although E-cadherin, β-catenin, and actin are uniformly distributed throughout the epithelia, relatively few organized adherens junctions are observed by EM between either boundary and/or interior SMG cells (Hieda *et al.*, 1996). With few intimate contacts, the interior cells are destined for disaster. With few exceptions (blood cells mainly), "lone rangers" do not survive (Ruoslahti, 1997).

A wide variety of growth factors, cytokines, and their cognate receptors are expressed in this cellular array (Table 2; Jaskoll and Melnick, 1999). A few are of particular interest. TNF is found throughout, but TNF-R1 and TNF-R2 are localized to central cell elements destined for initial apoptosis; IL-6 and IL-6R are also detectable. Recall that TNF-R1 and TNF-R2 binding promotes the up-regulation of cytokines such as IL-6 (Ashkenazi and Dixit, 1998). Up-regulation of the IL-6/IL-6R pathway has recently been shown to down-regulate adhesion molecule expression (Oh *et al.*, 1998). EGF, expressed for the first time in the *Canalicular Stage*, is mainly in more central cell elements as well, though EGF-R is present throughout the epithelia. EGF appears to induce the release of the E-cadherin/catenin complex from the cytoskeleton (Kemler, 1993). Thus, the EGF and IL-6 pathways may serve to disrupt what few adherens junctions might exist at a time coincident with apoptosis. The strategy: Isolate the cells and drive them to suicide.

In this regard, perhaps the most interesting of all is the presence and changing location of p53, a well-known tumor suppressor (Evan and Littlewood, 1998) and regulator of cell differentiation and development (Almog and Rotter, 1997). This transcription factor is normally maintained in reserve at low levels through interaction with the Mdm-2 protein that signals its degradation (Chen *et al.*, 1994; Evan and Littlewood, 1998). DNA damage-induced phosphorylation of either p53 or Mdm-2 prevents the two proteins from interacting, thus stabilizing and activating p53. There are two primary cellular responses to p53 activation, cell cycle arrest and apoptosis. Cell cycle arrest occurs in the $G_1 \rightarrow S$ and $G2 \rightarrow M$ transitions and is effected by up-regulation of p21, a cyclin-dependent kinase (Cdk) inhibitor (Bunz *et al.*, 1998). While less clear, p53 appears to promote apoptosis by the modulation of specific target genes, including the Bcl-2 antagonist Bax (Yin *et al.*, 1997), IGF-IR (Prisco *et al.*, 1997), and the binding protein IGF-BP3 (Buckbinder *et al.*, 1995). p53 mRNA expression has been detected at high levels in mouse SMG epithelium beginning at E14.5 (*Pseudoglandular Stage*); it is greatly reduced by E18.5 (*Late Terminal Bud Stage*) (Schmid *et al.*, 1991). Our studies reveal that p53 has its most telling distribution in the *Canalicular Stage*. At the outset, p53 protein is localized in nearly all the epithelial cells, frequently being absent from those at the array boundary (Fig. 12C). This may be indicative of a response in cells made unhealthy by the absence of anchorage (Ruoslahti, 1997), or it may simply be indicative of normal embryonic control of cell division (Campagne and Gill, 1998), or both. With the initiation of apoptosis from the center to the periphery, p53 is concentrated to and marks the next consecutive concentric layer of cells targeted for death (Fig. 13D). Perhaps this pattern also explains the declining presence of IGF-IR in these interior cells and the near-absence of its growth promotion ligand, IGF-II (Jaskoll and Melnick, 1999). Ligand-activated IGF-IR protects cells from apoptosis (Resnicoff *et al.*, 1995; Sell *et al.*, 1995). p53 protein causes a decrease in IGF-IR levels by repressing transcription from the *Igf1r* gene promoter; declining IGF-IR levels and the near-absence of ligand are permissive for apoptosis (Prisco *et al.*, 1997).

Finally, there remain two key puzzles: (1) Why does apoptosis radiate over time from the center to the periphery? and (2) What is the stop signal for this canalization "game"? Answers to these questions are entirely specula-

tive, but the "black box" plea is hardly satisfying. Relying upon Wainwright (1988), among others, let us instead look to the mechanical properties of our stylized sphere and cylinder with its cross-sectional hexagonal array (Figs. 12, 13). Only this time, think of it as a specially shaped balloon which you have filled with oil and water. Biomaterials are fluid and as such have a wide range of mechanical properties that are difficult to predict, not least because these properties depend on the substructure of their constituent materials. Regardless, every force applied to biomaterials will cause a deformation. Deformability is related to the low viscosity of water and lipid in the biomaterials. Our specially shaped balloon is thus a hydrostatic-like system in which the rubber membrane may be analogized to the boundary cells whose basal surfaces face, and anchor for integrity to, the ECM.

The boundary cell layer is in tension because it encloses and compresses the internal fluid cell contents; in turn, the compressed fluid cell volume produces tension upon the boundary cell layer. In the sphere, stress in the boundary cell layer will be in all directions and the same everywhere. In the cylinder, stress exerted by the contents on the circumference will be twice that exerted on the length, assuming the system is closed. Thus, compression and tension will differ between components of the branch/bud structure. Also, if we return to our view of the cross-sectional hexagonal array (Fig. 12B), the compression will be greatest at the center. Finally, compression and tension pressures will change over time as internal cells die and are replaced by an amorphous fluid.

Much work is now being done on the mechanical control of gene expression and regulation (*e.g.*, Torday *et al.*, 1998). Much of this work is related to the phenomenon of anchorage dependence and the cause of anoikis (Chen *et al.*, 1997; Glanz, 1997; Huang *et al.*, 1998). Results suggest that changes in the balance of mechanical forces between integrins and the cytoskeleton control downstream signaling cascades. Integrin, as noted above, is the molecular bridge between the ECM and the actin cytoskeleton. This system appears to integrate mechanical signals associated with changes in cell shape (*e.g.*, stretch), with chemical signals elicited directly by integrin binding, and thus modulate gene expression. Further, ECM anchoring seems to promote cell stability by resisting contractile forces transmitted across integrins, thus mechanically stabilizing the nucleo-cytoskeletal lattice. Finally, the increased flexibility of the cytoskeleton that has been observed in non-anchored, rounding cells is thought to permit the occurrence of intracellular structural re-arrangements that are lethal, including the nuclear structural degeneration that characterizes apoptosis.

And now the two puzzles: *Query* 1: Why does apoptosis radiate over time from the center to the periphery? *Answer*: Compression force is greatest at the center → mechanotransduction → EGF, TNF-R, and IL-6/IL-6R up-regulation → loss of adherens junctions and contact with neighbors → p53-mediated cell death. (This scenario repeats itself in each subsequent concentric cell layer in each subsequent generation, mechonotransduction being initiated by inwardly directed compression force and outwardly directed tension force.) *Query* 2: What is the stop signal for this canalization "game?" *Answer*: As we noted, boundary epithelial cells face the ECM and are bound to it, thereby preserving their survival signals. When apoptosis claims the last interior cells, there is a subtle change in boundary cell geometry and an important re-organization of intercellular junctions. In addition to adherens junctions, desmoplakins I/II (desmosomes) and ZO-1 (tight junctions) appear (Hieda *et al.*, 1996). This ensures survival, initiates the morphogenesis of the apical-basal polarized epithelial cell type which is crucial to its destined function (Rodriguez-Boulan and Nelson, 1989), and marks a sudden discontinuity from prior gradually changing conditions to one which terminates lumen formation. In sum, answers to these two queries are the bookends of a process which begins with a mass of only one cell type (*initial condition*) and concludes with two, a sheet of polarized epithelial cells and the ghosts of their erstwhile neighbors (*equilibrium state*). In between lie the transient states modeled by the canalization "game", a convenient visualization of a dynamic system whose rate of change is a function of time and of system parameters.

(V) *Concluding Remarks:* *Developmental Biocybernetics*

There is information and there is information processing. This essay contains information coded in letters at one level and, at a higher level, in agreed-upon meanings of words, syntax, and grammar. It will stay with me unless I send these codes to you by some means, electronic or otherwise. Thus, there is an important difference between information coded in DNA and transfer of that information to sites elsewhere, between storing Chopin's music in your head and transferring its beauty by piano to my auditory system with some fidelity.

Nearly four decades ago, Maruyama (1963) clearly outlined the developmental biologist's nightmare:

> "[I]t is not necessary for the genes to carry all the information regarding the adult structure, but it suffices for the genes to carry a set of rules to generate the information.
>
> "The amount of information to describe the resulting pattern is much more than the amount of information to describe the generating rules and the positions of the initial tissues. The pattern is

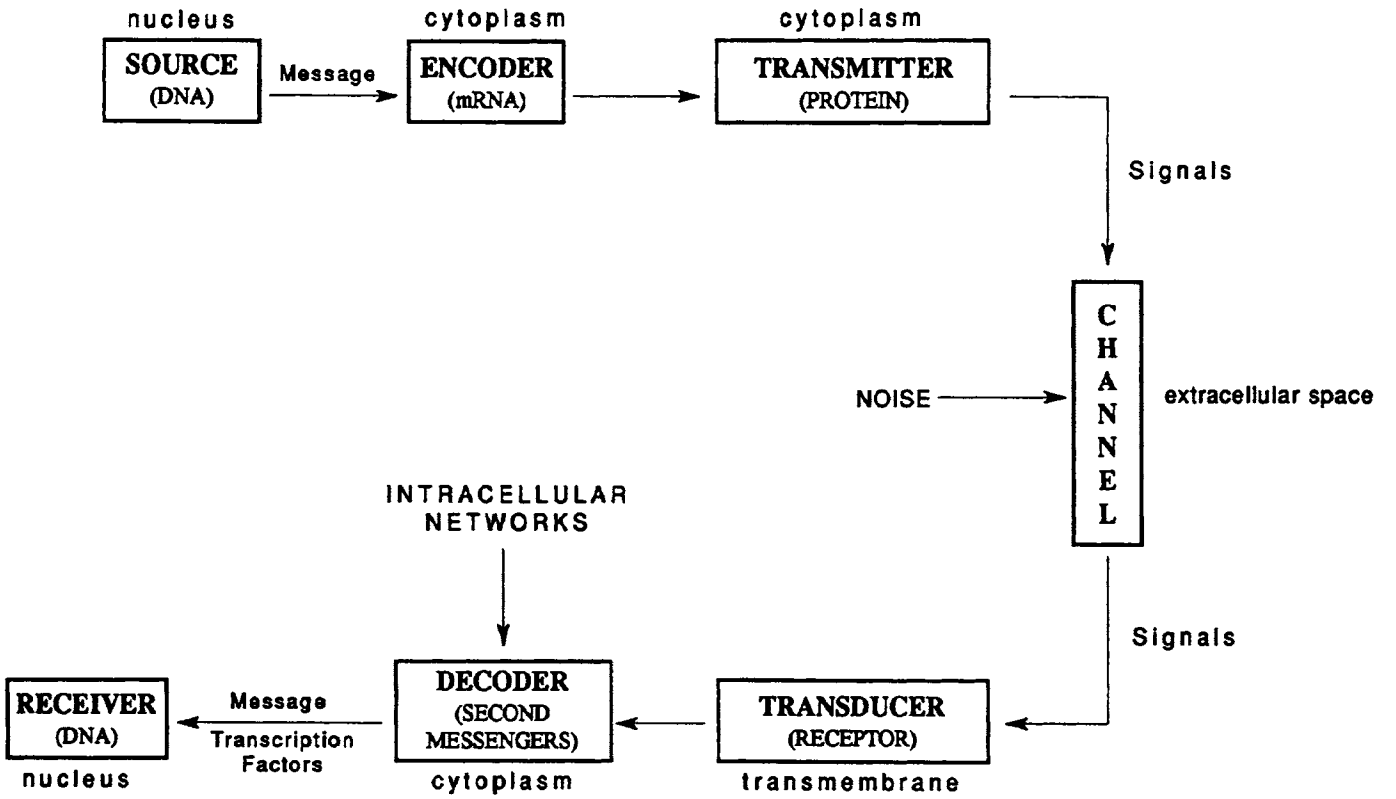


Figure 14. Biocybernetics: adapting information theory. Details are found in the text.

generated by the rules and by the *interaction* between the tissues. In this sense, the information to describe the adult individual was not contained in the initial tissues at the beginning but was generated by their interactions.

"[I]t is in most cases impossible to discover the simple generating rules after the pattern has been completed, except by trying all possible sets of rules. When the rules are unknown, the amount of information needed to discover the rules is much greater than the amount of information needed to describe the rules. This means that there is much more waste, in terms of the amount of information, in tracing the process backwards than in tracing it forward."

Using the lumen problem above, we can see that the best we can do is guess at the rules after observing the event closely—a grand tautology. Nevertheless, though the rules (program) may forever be obscure, we can make considerable progress in understanding the transfer of information by mechanical and chemical signal transduction if we place it in the context of an adapted Information Theory (Fig. 14). Fifty years ago, Shannon and Weaver (1949) presented a mathematical theory of how information in the form of a message or signal from a SOURCE transmitted to a RECEIVER is influenced by the CHANNEL through which that signal must pass. They concluded that, in a closed system in which nothing comes in from the outside, there can never be more information presented to the RECEIVER than was initially signaled by the SOURCE. In fact, given that there is almost always "noise" in the CHANNEL, there is likely to be less information presented to the RECEIVER unless compensated for by repeated signals of the same type.

We can adapt this general scheme (Fig. 14) to accommodate our current knowledge (Fig. 7; Table 2). Several features of Fig. 14 are worth noting. First, and most important, the system is necessarily *open*. It is made so by intracellular networking, and so the RECEIVER may be presented with more information than was initially signaled by the SOURCE. Second, "noise" in this context is comprised of the vagaries of extracellular space. As such, the efficiency of the signal transmission is reduced and a general redundancy of signal is a given. Finally, as the complexity of the system reveals itself in the years ahead, our ability to make highly precise statements about its "real-world" behavior will continue to diminish. This will be compensated for by an increasing capacity to make a larger number of fuzzy statements that are *proximate* to the "real-world" and thus provide better understanding (Kosko, 1993). Clever functional studies and closer collaboration with our mathematics colleagues will fill in the blanks.

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References

Agrawal M, Brauer PR (1996). Urokinase-type plasminogen activator regulates cranial neural crest cell migration in vitro. *Dev Dyn* 207:281-290.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994). Molecular biology of the cell. 3rd ed. New York: Garland Publishing.

Almog N, Rotter V (1997). Involvement of p53 in cell differentiation and development. *Biochim Biophys Acta* 1333:F1-F27.

Antonelli PL, Rogers TD, Willard MA (1973). Geometry and the exchange principle in cell aggregation kinetics. *J Theoret Biol* 41:1-21.

Ashkenazi A, Dixit VM (1998). Death receptors: signaling and modulation. *Science* 281:1305-1308.

Ball WD, Hand AR, Moreira JE (1991). A neonatal secretory protein associated with secretion granule membranes in developing rat salivary glands. *J Histochem Cytochem* 39:1693-1706.

Bhalla US, Iyengar R (1999). Emergent properties of networks of biological signaling pathways. *Science* 283:381-387.

Buckbinder L, Talbott R, Valasco-Miguel S, Takenaka I, Faha B, Selzinger BR, *et al.* (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* 377:646-649.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, *et al.* (1998). Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. *Science* 282:1497-1501.

Campagne MVL, Gill R (1998). Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21, p27, p57, p16, cyclin G1, and the proto-oncogene bax. *J Comp Neurol* 397:181-198.

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE (1997). Geometric control of cell life and death. *Science* 276:1425-1428.

Chen CY, Oliner JD, Zhan Q, Fornace AJ, Vogelstein B, Kastan MB (1994). Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc Natl Acad Sci USA* 91:2684-2688.

Clark EA, Brugge JS (1995). Integrins and signal transduction pathways: the road taken. *Science* 268:233-239.

Coppola D, Ferber A, Miura M, Sell C, D'Ambrosio C, Rubin R, *et al.* (1994). A functional insulin-like growth factor I receptor is required for the mutogenic and transforming activities of the epidermal growth factor receptor. *Mol Cell Biol* 14:4588-4595.

Cutler LS, Gremski W (1991). Epithelial-mesenchymal interactions in the development of salivary glands. *Crit Rev Oral Biol Med* 2:1-12.

Denny PC, Mirels L, Denny PA (1996). Mouse submandibular gland salivary apomucin contains repeated N-glycosylation sites. *Glycobiology* 6:43-50.

Denny PC, Ball WD, Redman RS (1997). Salivary glands: a paradigm for diversity of gland development. *Crit Rev Oral Biol Med* 8:51-75.

Engel ME, Datta PK, Moses HL (1998). Signal transduction by tranforming growth factor-β: a cooperative paradigm with extensive negative regulation. *J Cell Biochem* (Suppl 30/31):111-122.

Evan G, Littlewood T (1998). A matter of life and cell death. *Science* 281:1317-1322.

Galen (1979). On the natural faculties. Cambridge, MA: Harvard University Press.

Gierer A (1973). Molecular models and combinatorial principles in cell differentiation and morphogenesis. *Cold Spring Harbor Symp Quant Biol* 38:951-961.

Glanz J (1997). Force carrying web pervades living cell. *Science* 276:678-679.

Gresik EW, Kashimata M, Kadoya Y, Mathews R, Minami N, Yamashina S (1997). Expression of epidermal growth factor receptor in fetal mouse submandibular gland detected by a biotinyltyramide-based catalyzed signal amplification method. *J Histochem Cytochem* 45:1651-1657.

Hardman P, Spooner BS (1992). Localization of extracellular matrix components in developing mouse salivary glands by confocal microscopy. *Anat Rec* 234:452-459.

Hardman P, Landels E, Woolf A, Spooner B (1994). TGF-β1 inhibits growth and branching morphogenesis in embryonic mouse submandibular and sublingual glands in vitro. *Dev Growth Differ* 36:567-577.

Hieda Y, Iwai K, Morita T, Nakanishi Y (1996). Mouse embryonic submandibular gland epithelium loses its tissue integrity during early branching morphogenesis. *Dev Dyn* 207:395-403.

Huang S, Chen CS, Ingber DE (1998). Control of cyclin D1, p27, and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol Cell Biol* 9:3179-3193.

Jaskoll T, Melnick M (1999). 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-β2, TGF-β3 and EGF-R null mutations. *Anat Rec* 256:252-268.

Jaskoll T, Luo W, Snead ML (1998a). *Msx-2* expression and glucocorticoid-induced overexpression in embryonic mouse submandibular glands. *J Craniofac Genet Dev Biol* 18:79-87.

Jaskoll T, Chen H, Denny PC, Denny PA, Melnick M (1998b). Mouse submandibular gland mucin: embryo-specific mRNA and protein species. *Mech Dev* 74:179-183.

Jaskoll T, Choy HA, Melnick M (1994). Glucocorticoids, TGF-β, and embryonic mouse salivary gland morphogenesis. *J Craniofac Genet Dev Biol* 14:217-230.

Jones JI, Clemmons DR (1995). Insulin-like growth factors and their binding proteins: biologic actions. *Endocrinol Rev* 16:3-34.

Jowett AK, Vainio S, Ferguson MJW, Sharpe PT, Thesleff I (1993). Epithelial-mesenchymal interactions are required for *msx1* and *msx2* gene expression in the developing murine molar tooth. *Development* 117:461-470.

Kadoya Y, Kadoya K, Durbeej M, Holmvall K, Sorokin L, Ekblom P (1995). Antibodies against domain E3 of laminin-1 and integrin α6 subunit perturb branching epithelial morphogenesis of submandibular gland but by different modes. *J Cell Biol* 129:521-534.

Kashimata M, Gresik EW (1997). Epidermal growth factor system is a physiological regulator of development of the mouse fetal submandibular gland and regulates expression of the α6-integrin subunit. *Dev Dyn* 208:149-161.

Kauffman SA (1993). The origins of order. New York: Oxford University Press.

Kemler R (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet* 9:317-321.

Keski-Oja J, Blasi F, Leof EB, Moses HL (1988). Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-β. *J Cell Biol* 106:451-459.

Kosko B (1992). Neural networks and fuzzy systems. Englewood Cliffs, NJ: Prentice-Hall.

Kosko B (1993). Fuzzy thinking. The new science of fuzzy logic. New York: Hyperion.

Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, *et al.* (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* 69:927-938.

LeRoith D, Werner H, Beitner-Johnson D, Roberts CT (1995). Molecular and cellular aspects of insulin-like growth factor I receptor. *Endocrinol Rev* 16:143-163.

Lin HH, Stubbs LJ, Mucenski ML (1997). Identification and characterization of a seven transmembrane hormone receptor using differential display. *Genomics* 41:301-308.

Liu YH, Liang MA, Wu LY, Luo W, Kundu R, Sangiorgi F, *et al.* (1994). Regulation of the Msx 2 homeobox gene during mouse embryogenesis: a transgene with 439 bp of 5′ flanking sequence is expressed exclusively in the apical ectodermal ridge of the developing limb. *Mech Dev* 48:187-197.

Macauley SP, Tarnuzzer RW, Schultz GS, Chegini N, Oxford GE, Humphreys-Beher MG (1997). Extra-cellular matrix gene expression during mouse submandibular gland development. *Arch Oral Biol* 42:443-454.

Mandelbrot BB (1983). The fractal geometry of nature. San Francisco: W.H. Freeman.

Maruyama M (1963). The second cybernetics: deviation-amplifying mutual causal processes. *Am Scient* 51:164-179.

Melnick M, Jaskoll T (1997). Steroid transduction pathway and growth factor mediated embryonic salivary gland development. In: Studies in stomatology and craniofacial biology. Cohen MM, Baum BJ, editors. Amsterdam: IOS Press, pp. 67-84.

Melnick M, Chen H, Buckley S, Warburton D, Jaskoll T (1998). Insulin-like growth factor II receptor, transforming growth factor-β, and Cdk4 expression and the developmental epigenetics of mouse palate morphogenesis and dysmorphogenesis. *Dev Dyn* 211:11-25.

Melnick M, Chen H, Zhou Y, Jaskoll T (2000). Interleukin-6 signaling and submandibular salivary gland morphogenesis. *Cells Tissues Organs* (in press).

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pederson RA, Werb Z, *et al.* (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 376:337-341.

Mina M, Gluhak J, Upholt WB, Kollar EJ, Rogers B (1995). Experimental analysis of msx-1 and msx-2 gene expression during chick mandibular morphogenesis. *Dev Dyn* 202:195-214.

Moreira JE, Ball WD, Mirels L, Hand AR (1991). Accumulation and localization of two adult acinar cell secretory proteins during development of the rat submandibular gland. *Am J Anat* 191:167-184.

Nakanishi Y, Morita T, Nogawa H (1987). Cell proliferation is not required for the initiation of early cleft formation in mouse embryonic submandibular epithelium in vitro. *Development* 99:429-437.

Odekon LE, Blasi F, Rifkin DB (1994). Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-β to TGF-β. *J Cell Physiol* 158:398-407.

Oh JW, Van Wagoner NJ, Rose-John S, Benveniste EN (1998). Role of IL-6 and the soluble IL-6 receptor in inhibition of VCAM-1 gene expression. J *Immunol* 161:4992-4999.

Orr-Urtreger A, Lonai P (1992). Platelet-derived growth factor-A and its receptor are expressed in separate, but adjacent cell layers of the mouse embryo. *Development* 115:1045-1058.

Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, *et al.* (1998). An essential role for ectodomain shedding in mammalian development. *Science* 282:1281-1284.

Prisco M, Hongo A, Rizzo MG, Sacchi A, Baserga R (1997). The insulin-like growth factor I receptor as a physiologically relevant target of p53 in apoptosis caused by interleukin-3 withdrawal. *Mol Cell Biol* 17:1084-1092.

Ransom R (1981). Computers and embryos. Models in developmental biology. New York: John Wiley & Sons.

Resnicoff M, Abraham D, Yuntanawiboonchai W, Rotman H, Kajstura J, Rubin R, *et al.* (1995). The insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. *Cancer Res* 55:2463-2469.

Rodriguez-Boulan E, Nelson WJ (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* 245:718-725.

Ruoslahti E (1997). Stretching is good for a cell. *Science* 276:1345-1346.

Schmid P, Lorenz A, Hameister H, Montenarh M (1991). Expression of p53 during mouse embryogenesis. *Development* 113:857-865.

Sell C, Baserga R, Rubin R (1995). Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. *Cancer Res* 55:303-306.

Shannon C, Weaver W (1949). The mathematical theory of communication. Urbana, IL: University of Illinois Press.

Sonnenberg E, Meyer D, Weidner M, Birchmeier C (1993). Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J *Cell Biol* 123:223-235.

Spooner BS, Bassett KE, Spooner BS Jr (1989). Embryonic salivary gland epithelial branching activity is experimentally independent of epithelial expansion activity. *Dev Biol* 133:569-575.

Strohman RC (1997). The coming Kuhnian revolution in biology. *Nature Biotech* 15:194-200.

Takeichi M (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251:1451-1455.

Torday JS, Sanchez-Esteban J, Rubin LP (1998). Paracrine mediators of mechanotransduction in lung development. *Am J Med Sci* 316:205-208.

Vignon F, Rochefort H (1992). Interactions of pro-cathepsin D and IGF-II on the mannose-6-phosphate/IGF-II receptor. *Breast Cancer Res Treat* 22:47-57.

Visser JA, Themmen APN (1998). Downstream factors in transforming growth factor-β family signaling. *Mol Cell Endocrinol* 146:7-17.

von Neumann J (1966). The theory of self-reproducing automata. Urbana, IL: University of Illinois Press.

Waddington CH (1957). The strategy of the genes. London: George Allen & Unwin, pp. 11-58.

Wainwright SA (1988). Axis and circumference. Cambridge, MA: Harvard University Press.

Wessells NK (1977). Tissue interactions and development. Menlo Park, CA: Benjamin Cummins.

Williams T, Bjerknes R (1972). Stochastic model for abnormal clone spread through epithelial basal layer. *Nature* 236:19-21.

Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T (1997). Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. *Nature* 385:637-640.