A hierarchy of ECM-mediated signaling tissue-specific gene expression regulates tissue-specific gene expression

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A dynamic and reciprocal flow of information between cells and the extracellular matrix contributes significantly to the regulation of form and function in developing systems. Signals generated by the extracellular matrix do not act in isolation. Instead, they are processed within the context of global signalling hierarchies whose constituent inputs and outputs are constantly modulated by all the factors present in the cell's surrounding microenvironment. This is particularly evident in the mammary gland, where the construction and subsequent destruction of such a hierarchy regulates changes in tissue-specific gene expression, morphogenesis and apoptosis during each developmental cycle of pregnancy, lactation and involution.

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Introduction

There is no longer any doubt that interactions between cells and the extracellular matrix (ECM) initiate a flow of information that acts to regulate many fundamental processes throughout development. These include cell migration in the early embryo, morphogenesis during organ formation, and the modulation of growth and differentiation programs of many, if not all, specialized cell types [1,2]. Many interactions between ECM ligands and cell surface receptors have been well characterized, and careful molecular dissections using simplified tissue culture models have identified a number of the signals initiated by these interactions [3,4,5**]. The task now at hand is to determine the biologically relevant aspects of this information flow, particularly as they pertain to the development of function as well as form in complex systems.

The most thoroughly studied interactions between the cell and the ECM are mediated by the integrins, a family of heterodimeric transmembrane receptors composed of more than 20 functional members, many of which have overlapping and competing affinities for individual ECM ligands [3]. For example, specific integrin-binding motifs are often found in a number of ECM molecules, and many integrins have affinity for more than one ECM ligand. In addition, individual ECM molecules can be either adhesive or anti-adhesive, stimulate migration or morphogenesis, and promote growth or differentiation [o-10]. Such widespread differences in functional response may occur because one ECM ligand can activate a number of integrins [11,12]. However, the overwhelming body of experimental evidence to date indicates that many of the signals initiated are similar, regardless of the ligand or the integrin involved [4,5”]. In addition, gene ablation studies in transgenic mice indicate that the signals generated by individual ligands and integrins appear to be complementary and redundant [13,14*]. Therefore, it is not unreasonable to postulate that it is the signal processing rather than signal generation that is most important in determining the specific phenotypic response to a particular ECM-integrin interaction.

Some years ago, we hypothesized that information flow between cells and the ECM is both dynamic and reciprocal [15]. In this model, physical connections between the ECM, cytoskeleton and the nucleus occur along a structural continuum which serves as an architectural scaffold upon which biochemical signal transduction pathways are overlaid [lb]. Thus, the context of ECM ligand presentation, accessory factors present in the microenvironmental milieu, and the differentiation state of the responding cell al impinge upon a hierarchical signalling template to regulate both input and output [17-191. Nowhere is the importance of this finely tuned signal processing more apparent than in the mouse mammary gland, where epithelial cells and the surrounding ECM act together as a functional unit throughout development. In the resting gland, modifications of the ECM influence ductal branching, end-bud development and epithelial proliferation [20]; during pregnancy and lactation, an intact basement membrane is required for the emergence of differentiated function [21]; and finally, after weaning, degradation of the ECM by metalloproteases [22] triggers the massive programmed cell death that occurs during involution, an event which brings the gland back to its original resting state [23,24’*].

In this review we will briefly describe how .ECM-mediated signals are generated within the broad categories of adhesion, architecture, and biochemical signalling. Examples will then be provided to demonstrate how the processing of these signals affects specific aspects of cell

function in a variety of systems. Finally, using data gleaned from specialized three-dimensional tissue culture models and direct experimental manipulations in viva, this information processing will be placed within the context of a dynamic signalling hierarchy that is constructed and dismantled during each cycle of mammary gland development.

Adhesion, architecture and gene expression

ECM-cytoskeleton linkages

Reduced to its bare essentials, the interaction between cells and the ECM is adhesive. In monolayer culture, integrins cluster within 'focal adhesions' at sites of close contact between the cell and the ECM [25]. Once there, the integrin cytoplasmic domains recruit a number of structural proteins to a developing adhesion complex, thereby generating a physical link with the actin-based cytoskeleton [5**,26]. Although minor, cell-specific differences in adhesion complex composition have been noted [27,28], there is, as yet, little evidence that adhesion complex composition is regulated in a ligand- or integrin-specific manner. In addition, there appears to be functional redundancy within the complex itself. Thus, if the ubiquitous actin-binding protein vinculin is ablated genetically, ECM-mediated adhesion occurs, complexes form, actin stress fibres radiate from the complexes, and cell spreading is initiated, although with somewhat reduced efficiency [29].

Despite the redundancy and overall lack of specificity in adhesion complex formation, ECM-mediated linkage to the cytoskeleton can profoundly alter cell morphology and function [30]. For example, when hepatocytes are maintained on a rigid ECM, the cells spread, lose differentiated function and proliferate in the presence of appropriate growth factors [31]. Alternatively, when they are plated on a malleable ECM, they round-up, differentiation-related genes are expressed, and proliferation does not occur [32,33]. Interestingly, when cell rounding is initiated by plating hepatocytes on an inert, non-adhesive substratum, differentiation ensues in the complete absence of exogenously added ECM [34,35]. However, under the latter conditions, the cells aggregate and deposit an endogenously produced ECM. Thus, experiments using single cells were required before it could be stated definitively that changes in cell shape, rather than specific signals generated by a particular ECM ligand, act as a switch between the proliferative and differentiative states [36,37"]. Similar cell shape dependent differentiation has been observed in a number of other cell types, including keratinocytes, steroidogenic cells, retinal pigmented epithelial cells and mammary epithelial cells [38-42].

ECM-mediated effects on transcription factors

How do ECM-mediated changes in cell shape initiate a switch between the proliferative and differentiative states? One way would be to alter the expression of transcription factor cascades that regulate this process. In the case of rounded hepatocytes maintained on a malleable ECM, expression of immediate early response genes such as jun, *fos* and myc is downregulated and differentiation of the cells occurs; in addition, the DNA-binding activity of transcription factors such as API, which serve as endpoints for proliferation-associated signal transduction in flattened cells, is attenuated [43"]. Simultaneously, expression of two liver-specific transcription factors, HNF~cx and eH-TF, both of which modulate the activity of an albumin gene enhancer,

is upregulated in a cell shape dependent manner [44]. Changes in the expression of factors such as these may also indirectly influence the differentiated state. More precisely, HNF3a, homologous to the product of the fork *head* gene in *Drosophila* , is likely to play a role similar to that elicited by homeotic proteins [45].

Homeobox-containing genes have been described as master regulators of cell fate and pattern formation in a number of developmental systems [46,47]. Despite the fact that little is known about the direct targets of homeotic proteins, various family members have been shown to regulate expression of ECM molecules, cell adhesion molecules, and components of cell-cell junctions, all of which are involved in morphogenetic processes ([48-501; see Cunningham, this issue, pp 628-633). The expression of the differentiation-associated CCAATT/enhancer binding proteins (C/EBPs) is also maintained, or upregulated, in rounded hepatocytes, such that individual family members interact to form heterodimers that bind to consensus sites present in the albumin promoter with functional consequences [43**,51]. Interestingly, C/EBP activity itself may regulate cell shape. For example, when UEBP-b is overexpressed in flattened fibroblasts, they round-up and initiate adipocyte-specific gene expression (S Farmer,personal communication). Therefore, cellular architecture and the expression of differentiation-promoting transcription factors are dynamically intertwined.

The role of the nuclear matrix

Signals mediated by cell shape may be processed by the nuclear matrix [52], a network of anastamosing proteinaceous fibres that bind to DNA at ‘matrix attachment regions’ along areas of active chromatin [52,53]. The nuclear matrix appears to be physically linked to the intermediate filament cytoskeleton via the nuclear lamins [54]. As integrin-mediated changes in cytoskeletal tension are af&cted by pharmacological disruption of intermediate filaments [55], ECM-dependent alterations in morphology may restructure the nuclear matrix. This could, in turn, initiate changes in DNA-binding activity and therefore in gene expression [56]. The latter appears to be the case, at least in part, in differentiating osteoblasts. As these cells shift horn a proliferative to a rounded, differentiated state, the composition of the nuclear matrix changes dramatically and a novel nuclear matrix protein, NMP-2, appears. NMP-2 binds to the promoter of the osteocalcin gene and induces its expression [57]. As an adjunct to affecting the DNA-binding activity of structural proteins, architectural changes to the nuclear matrix can also alter the sequestration state -and therefore binding ability-of a number of soluble transcription factors discussed above, including members of the APl and C/EBP families [58*].

Signalling

Protein phosphorylation is a common response to integrin activation in many cell types [4]. Although integrins themselves have no intrinsic enzymatic activity, the activation and recruitment of an associated tyrosine kinase, pp125FAK, to adhesion complexes helps initiate such biochemical signalling [59*]. However, ECMmediated activation of FAK (focal adhesion kinase) is not integrin subunit specific and FAK is also activated by a number of non-integrin receptors [60,61]. Thus, signal generation through FAK alone cannot be responsible for eliciting a specific phenotypic response. Instead, its many associations with the cytoskeleton, adaptor

proteins and other biochemical transduction moieties suggest that FAK acts to modulate multiple, intersecting transduction pathways [5**]. Other tyrosine kinases and a novel serine/threonine kinase (GE Hannigan et al., abstract 357 in MO/ Biol Cell 1994, vol 5s) are also probably involved. In fact, it is rapidly becoming apparent that integrin activation generates so many different signals that it may be impossible to directly link input to output. Instead, this signal cascade may be analogous to a gestalt, where the eventual output is greater than, or at least different fi-om, the sum of the individual inputs.

Cellular interactions with the ECM can also modulate signals initiated by soluble factors. In fibroblasts that are maintained in suspension, platelet-derived growth factor (PDGF) binds to its receptor and activates phospholipase C (PLC), yet there is little release of the PLC-dependent second messengers, diacylglycerol and inositol 1,4,5-trisphosphate [62]. However, when the cells are plated on fibronectin, PDGF-mediated signal transmission is restored as a result of an integrin-dependent production of phosphatidylinositol 4,5-bisphosphate, the phospholipid that is the appropriate substrate for PLC. In the case of signalling mediated by the fibroblast growth factor (FGF) receptor, ECM-dependent processing occurs much further down the transduction pathway. Thus, in suspended endothelial cells, FGF generates signals that induce the appropriate increased expression of early and late response genes that are required to drive the cells towards the proliferative state. Cell cycle progression is blocked, however, and DNA synthesis does not occur. Only when the cells are plated on a rigid ECM that allows spreading can they proceed through the cell cycle and proliferate [63*]. It has been suggested [64*]that these examples of signal processing require the integration of signals originating from the ECM and soluble ligands. As will be explained below, cellular architecture is probably responsible for much of this integration.

Cell spreading on a rigid ECM is associated with the formation of actin stress fibres, a process which depends on the activation of the small GTP-binding protein, rho [65]. Conversely, chronic activation of another GTP-binding protein, GAP, induces stress fibre breakdown, apparently by feeding back to inhibit rho [66]. Thus, under certain conditions, architectural and biochemical signals appear to intersect, because GAP also modulates the ubiquitous ~21 rar-mediated transduction pathway. FAK, through its interactions with other proteins that localize to the adhesion complex (e.g. kinases, the structural protein paxillin, and proteins containing src homology [SH2/SH3] domains), also impinges upon ras-dependent signalling, the ultimate effect of which may be stimulation of the downstream effector mitogen-activated protein (MAP) kinase [67-69]. Given the latter's ability to modulate transcription factor activity, its stimulation provides a direct link between integrins and the regulation of gene expression [70]. This pathway appears to be stimulated by many factors, however, and given the kinetics of its activation, it may occur in a FAK-independent fashion under some circumstances [67,71*]. Thus, in the absence of appropriate signal processing there will be little or no specificity to the response. One way to ensure that the appropriate processing does indeed occur in the tissues is to construct a tissue-specific signalling hierarchy during the course of development.

Tissue-specific signalling hierarchies

We have attempted to resolve the apparent paradox of redundant signals eliciting specific responses by examining a broad range of ECM-dependent phenotypes in mouse mammary epithelial cells. To do so, a number of specialized three-dimensional tissue culture models were developed and a variety of phenotypes were manipulated in viva. This integrated approach enabled us to do reductionist experiments which examined individual aspects of differentiation in an appropriate developmental setting. As a result, we now conclude that the correct processing of ECM-generated signals requires the construction of a dynamic signalling hierarchy. The components of this hierarchy include: a titration of cell shape dependent changes in cytoskeletal and nuclear architecture; integrin-dependent biochemical signals that lead to the activation of ECM-responsive elements; reciprocal signals that travel outward from the nucleus back to the cell surface; and changes to the composition of the ECM itself. In addition, signals generated by accessory, non-ECM components present within the tissue microenvironment continuously modulate hierarchical setpoints. What follows is an outline of both the construction and destruction of this hierarchy during each developmental cycle of pregnancy, lactation, and involution in the mammary gland and our model system in culture (Fig 1).

Influence of cell shape

Almost all aspects of mammary epithelial cell differentiation are influenced by the extracellular matrix, specifically the surrounding basement membrane [18]. As is the case for differentiating hepatocytes, this substratum, or its individual components must be presented to the mammary epithelial cell in a malleable form, presumably so that ECM-dependent changes in shape can occur [38,72-741. In an effort to definitively separate the differentiative effect of changes in cell shape from those induced by ECM-mediated signalling, we recently isolated a mammary epithelial cell clone that is unable to deposit an endogenous ECM [75]. In these cells, rounding alone, which provides mechanical signals that profoundly alter nuclear architecture, leads to expression of the iron-binding milk protein, lactoferrin (MJ Close et al., abstract 381 in A4ol Biol Cell 1994, vol 5s; [76*]). This differentiative event appears to be analogous to albumin expression in hepatocytes because it does not require the presence of a specific ECM ligand, and it is associated with the downregulation of the APl transcription factor activity (CD Roskelley, MJ Bissell, unpublished data), a nuclear signal associated with the proliferative state [43**]. Therefore, mechanical signals whose processing requires an intact cytoskeleton [77,78], initiate gene expression even at this early stage of the mammary-specific signalling hierarchy.

Regulation of β-casein gene expression

Cells that are forced to round purely by mechanical means are ‘primed’ to move to the next level of the signalling hierarchy Thus, a flexible basement membrane overlay or even purified laminin alone added in solution, generates biochemical signals and a tissue-specific response, expression of the milk protein geneβ-casein [76*,79*]. Signal generation requires functional βl integrins, and it is associated with the formation of novel adhesion complexes, increased tyrosine phosphorylation and the activation of pp125FAK [19,80]. Importantly, signal generation is not cell shape dependent: all of the above signals are initiated in both flat and rounded cells, but only

the latter express β-casein [19,76*]. Therefore, differential signal processing and not signal generation is likely to account for this second tier of responsiveness of rounded, primed cells.

When added in solution to flat monolayers, laminin will eventually induce mammary epithelial cell rounding and β-casein expression. However, this process is slow, often requiring several days [75,76*,79*], and is dependent on α6 integrin (CD Roskelley, MJ Bissell, unpublished data). In contrast, ECM-mediated signals are processed within a few hours in pre-rounded cells and β-casein expression does not require functional α6 integrins [80]. These data suggest that different integrin subunits may mediate kinetically separable responses to the same ECM ligand. The initial response is morphological and is associated with the expression of lactoferrin, the first tier of the hierarchy in our model system. The second response occurs in a morphology-independent manner and it is associated with expression of β-casein, the second tier of hierarchy. Differences in functional response which rely on the activation of specific integrin subunits have been noted in other systems [81-83]. Furthermore, individual integrin subunit activation can lead to differential signal processing at the level of kinase substrate specificity [84,85]. It should be noted, however, that it is only the second tier β-casein signal that is sensitive to kinase inhibition [19,76*].

Differential second tier signal processing also occurs in the nucleus. In rounded, primed mammary epithelial cells, laminin induces a transient increase in the activity of APl family transcription factors just before the induction of β-casein expression (CD Roskelley, C Hauser, MJ Bissell, unpublished data). Interestingly, in non-responsive flattened cells, the initial APl activity is high, and no laminin-dependent transient increase is observed. This differential processing occurs despite the fact that FAK and MAP kinase are activated in both round and flat cells ([19]; CD Roskelley, MJ Bissell, unpublished data). Therefore, cell shape may act to regulate the 'setpoint' for FAK/MAP kinase dependent changes in transcription factor activity. In this way, the kinetically controlled activation of a transcription factor that is usually associated with the proliferative state (i.e. APl) could be translated into a functional signal that is involved in the differentiative process.

Insulin acts as a growth factor when mammary epithelial cells are maintained in an undifferentiated state. When ECM is added, however, insulin does not interfere with second tier β-casein signalling [86,87], suggesting that signals generated by the ECM may impinge upon insulin-dependent signal transduction. Recently, it has been demonstrated that integrin-mediated signals can influence events that occur downstream of the insulin receptor [88]. As insulin stimulates the rasdependent MAP kinase pathway, it will be important to determine if cellular interactions with the ECM prevent proliferative signals from interfering with second tier differentiative events. If this is indeed the case, such transduction pathway cross-talk might involve a rearrangement of the cell shape dependent 'setpoint' in a fashion similar to the one discussed above for APl activity.

ECM-responsive elements

Changes in cell shape may modulate transcription factor activity by influencing the structure and composition of the insoluble nuclear matrix, without affecting the levels of transcription factors. For example, a 160 bp enhancer, designated BCE-1, which was isolated from the bovine β-casein

gene is activated when mammary epithelial cells are placed on reconstituted basement membrane gels [86,87]. Like the promoter of the albumin gene, BCE-1 contains a G'EBP-binding element that is required for ECM-dependent activation. And yet, despite the fact that the expression of various UEBP isoforms is modulated during mammary gland development in viva, [So], soluble nuclear UEBP binding activity is not regulated by the ECM in culture (C Schmidhauser, C Myers, MJ Bissell, unpublished data). Thus, second tier signal processing may affect the ability of soluble transcription factors to load onto ECM-responsive elements, rather than their expression.

The ECM-response element BCE-1 is only appropriately regulated when it is stably integrated into the cellular genome (C Myers, CD Roskelley, MJ Bissell, unpublished data). The mouse mammary tumor virus (MMTV) enhancer is also affected by stable integration and it is regulated by ECM in mammary epithelial cells [go]. In viva, the interaction of the glucocorticoid receptor with the glucocorticoid response element of MMTV leads to changes in chromatin organization that alter nucleosome positioning and transcription factor loading, resulting in transcriptional activation [91,92]. Like the MMTV enhancer, activity of BCE-1 can be associated with the binding of the homeotic protein, HNF3α [94].

Second tier signal processing also affects the loading of transcription factors that respond to signals generated by prolactin, a lactogenic hormone that is absolutely required for ECM-dependent β-casein expression and BCE-1 activation [87]. Although signal transduction mediated by the prolactin receptor in general is not cell shape or ECM dependent (J Ashkenas, Z Werb, MJ Bissell, unpublished data), its ability to induce milk protein gene expression may be. The latter requires the activation of mammary gland factor (STAT 5) [95,96], which is a member of a family of transcription factors known as signal transducing activators of transcription. STAT-5 interacts with the activated prolactin receptor and is phosphorylated by a receptor-associated kinase. It then travels to the nucleus where it binds to a consensus sequence present in the promoters of milk protein genes, resulting in their transcriptional induction [97]. Importantly, STAT-5 binding to the β-lactoglobulin gene promoter appears to be ECM-dependent (C Streuli, CJ Watson, personal communication). BCE-1 itself contains a STAT-5 consensus binding site; however, mutational analysis of BCE-1 suggests that although the STAT-5 binding is necessary for ECM-dependent activation, it is not sufficient (C Schmidhauser, C Myers, MJ Bissell, unpublished data) [93]. Thus, further combinatorial signal processing must be required, and this probably involves the C/EBP-binding site, as it too is necessary, but not sufficient, for ECM-dependent BCE-1 activation.

Cell-mediated changes in ECM

Second tier signal processing can also trigger reciprocal, cell-mediated changes in the composition of the ECM itself. When mammary epithelial cells are isolated from mid-pregnant mice and placed on a rigid substratum of stromal collagen, they flatten, de-differentiate and stop producing β-casein. In contrast, when stromal collagen is presented in a malleable form, the cells round-up and β-casein expression is once again initiated [38,72]. We now know that the cells do not respond directly to signals generated by the collagen itself. Instead, changes in cell shape and polarity lead to the deposition of a laminin-containing basement membrane that in turn induces β-casein expression [790,98]. This deposition

also results in decreased ECM gene expression, an event that is regulated by the 'matrix modifier' transforming growth factor-β (TGF-β) [99]. Interestingly, basement membrane-second tier signal processing itself leads to the downregulation of TGF-β.

Morphogenesis

When single mammary epithelial cells are placed within basement membrane gels, second tier β-casein expression is induced despite the fact that the cells do not become polarized [80]. However, a third tier of the signalling hierarchy can be induced by placing mammary epithelial cells on top of the gel. Under these conditions the cells aggregate and self-assemble to form three-dimensional structures that resemble the alveoli in *vim* [73]. Once formed, these structures begin to secrete milk proteins vectorially into a central lumen, a process that requires the formation of junctional complexes between the cells [74,100]. The latter morphogenetic event is, at least in part, mediated by hydrocortisone, a lactogenic hormone that augments milk protein gene expression [72], induces tight junction formation [loll and increases basement membrane deposition (CD Roskelley, MJ Bissell, unpublished data).

ECM-dependent morphogenesis is associated with a complex post-transcriptional regulation of whey acidic protein (WAP) [102], a significant component of mouse milk. Although the signals required for WAP expression are architectural in nature, they can be modified in a dynamic and reciprocal fashion by soluble factors present in the surrounding microenvironment [102,103]. Thus, third tier signalling downregulates the expression of TGF-α, a growth factor that inhibits WAP expression and prevents the formation of polarized three-dimensional structures in culture [1031. Another growth factor, hepatocyte growth factor (HGF), may also influence third tier signalling by inducing ductal branching when mammary epithelial cells are placed within collagen gels [104]. Data such as these suggest that ECM-dependent regulation of growth factor production may act in concert with morphogenetic factors such as homeotic proteins to regulate both form and function.

Preliminary evidence indicates that the expression of homeobox-containing genes is modulated by exposure to ECM in mammary epithelial cells in culture (A Srebrow, MJ Bissell, abstract 3109 in Proceedings of the American Association for Cancer Research, 1995, vol 36). In addition, homeotic genes are differentially regulated during mammary gland development [105*], which suggests that their expression may also be influenced by basement membrane composition in vivo. Other classes of transcription factors may also act at this level in the signalling hierarchy. For example, forced expression of an inhibitor of the helix-loop-helix class of transcription factors leads to aberrant morphogenesis and the loss of expression of milk protein genes [106].

Interactions with the ECM not mediated by integrins also influence morphogenesis. For example, hyaluronic acid binds to the non-integrin receptor for hyaluronanmediated motility (RHAMM), an interaction that induces a short-lived phosphorylation of FAK that appears to destabilize focal adhesions as a prelude to cell migration [107]. Non-integrin ECM receptors can also influence the activity of growth factors associated with the ECM. Syndecan, a receptor with heparin sulphate proteoglycan side chains, binds to collagens, fibronectin and basic, fibroblast growth factor [108]. This heparin-dependent co-localization of ECM ligands and a growth factor results in the assembly of a novel signalling complex on the cell surface at morphogenetic boundaries in developing tissues [109]. In this vein, we have recently demonstrated that a domain of laminin

which binds to heparin is involved in the ECM-dependent differentiation of mammary epithelial cells [79*], although a direct involvement of proteoglycans has not been demonstrated as yet.

The association of laminin with other ECM molecules may also enhance differentiative signals. For example, the presence of entactin in solutions of laminin presented to mammary epithelial cells in culture reduces by tenfold the molar concentration of laminin required to induce p-casein expression compared to the threshold concentration required when pure laminin is added (J Ashkenas, CD Roskelley, Z Werb, MJ Bissell, unpublished data). This is probably explained by the ability of entactin to cross-link laminin, which produces supramolecular structures that are required to initiate integrin clustering and subsequent biochemical signalling [l lo]. Such ECM ligand cross-linking appears to have functional significance in vivo, as metalloprotease destruction of the basement membrane, an event that triggers the loss of differentiated function (see below), is initiated by the proteolytic digestion of entactin (CM Alexander, EW Howard, CJ Sympson, MJ Bissell, Z Werb, unpublished data).

Involution and ECM-dependent apoptosis

Endogenously produced metalloprotease activity mediates the destruction of the basement membrane after weaning and triggers the complete dismantling of the signalling hierarchy that is built up to direct mammary epithelial differentiation during pregnancy and lactation. This process, known as involution, is associated with remodeling of the ECM, downregulation of tissue-specific gene expression, loss of epithelial integrity, and a massive apoptosis that clears the gland of epithelial cells. The end result is a return to the resting, non-functional state which is characterized by a repopulation of the gland by stromal cells [22,23]. Early in involution, laminin and type IV collagen are destroyed whereas tenascin is deposited. Addition of the latter ligand to differentiated mammary epithelial cells in culture downregulates endogenous β-casein expression [111,112] and leads to programmed cell death (N Boudreau, PL Jones, MJ Bissell, unpublished data).

Many anchorage-dependent cell types undergo apoptosis when they are maintained in suspension, a process that is prevented by an integrin-mediated adhesion to and spreading on a rigid substratum [113-l 151. In tissues, additional steps are probably required to maintain cellular viability, Mammary epithelial cells continue to undergo apoptosis even when they are flat and well spread on different ECM components [24”]; apoptosis is prevented only if proper morphogenetic events take place. That the basement membrane itself is responsible is confirmed by the fact that the apoptotic process can be re-initiated by the forced expression of activated stromelysin-1 which mediates the destruction of the ECM [24**]. Loss of ECM results in upregulation of interleukin-converting enzyme (ICE), or an ICE-like homologue, which in turn leads to programmed cell death. Thus, the mechanisms that drive the integrindependent inhibition of apoptosis also have signaling hierarchies. Interestingly, in the involuting mammary gland, metalloprotease-dependent apoptosis is associated with a transient activation of APl transcription factor activity [116]. Therefore, once again, the use of similar, or perhaps even identical, signal transduction pathways elicits a specific phenotypic response, depending on the developmental stage of the gland and the signals present in the microenvironment.

The cyclical nature of ECM-dependent signalling in the mammary gland

In summary;the information flow between cells and the ECM which regulates the differentiation state of the mammary epithelium are integrated into a signaling hierarchy that is first constructed and then dismantled in a cyclical manner throughout development (Fig. 1). The first tier of the hierarchy involves mechanical signals associated with cell rounding that trigger the expression of the iron-binding protein, lactoferrin. Rounded cells, which come together, now deposit an endogenous basement membrane that leads to the second tier of the hierarchy, a laminin-specific induction of β-casein expression. This tier requires integrin-mediated biochemical signalling and transcription factor dependent enhancer activation. The third tier of the hierarchy relies upon ECM-mediated morphogenesis, wherein the presence of a basement membrane directs cell polarity, formation of a central lumen, vectorial secretion and the expression of WAF? Importantly, this hierarchy also appears to be operational during pregnancy. For example, lactoferrin can be detected early in pregnancy just as structural differentiation commences after the epithelial proliferation that characterizes the early portion of this developmental stage; β-casein is first detected in significant levels in mid-pregnancy, at a time when an important proportion of the cellular synthetic machinery becomes committed to milk protein production; and finally WAP is expressed late in pregnancy just before the onset of lactation. Upon the completion of lactation,the dismantling of this hierarchy that begins at weaning is mediated by ECM-degrading proteases, which act in a developmental stage specific manner to induce apoptosis. Thus, given that the ECM influences growth, differentiation and apoptosis, it may be unorthodox, but not surprising, that chronic metalloprotease expression leads to adenocarcinoma formation in the mammary gland (C Sympson et al., abstract 2493 in Mel ***Biol Cell 1994,*** vol ***5S),*** a process that is accompanied by an accelerated cycling of aberrant ECM-mediated signaling hierarchies.

Conclusions

Many of the molecular signals initiated by interactions between cells and the ECM have now been identified in simple experimental systems. However, because of the redundant, complementary, and modulatory nature of these signals, it has been difficult so far to identify which aspects of this information flow are functionally important. This is true even in transgenic models where the expression of individual ECM ligands and integrin subunits have been completely ablated. Thus, only by taking an integrated approach, which exploits both functional tissue-culture models and tissue-specific analyses in vivo, can the crucial components of cell-ECM interactions required for the generation of tissue form and function be elucidated. The mammary gland provides a useful model where such interactions play specific roles within a signalling hierarchy that directs cycles of growth, differentiation, morphogenesis and apoptosis throughout development.

Extracellular matrix emerges as the universal integrator of function in this tissue: a normal basement membrane maintains differentiation not only by specific signalling, but also by inhibiting growth, apoptosis and possibly carcinogenesis (Fig. 2).

Note added in proof

The data referenced in the text as C Streuli and CJ Watson, personal communication, is now in press [117].

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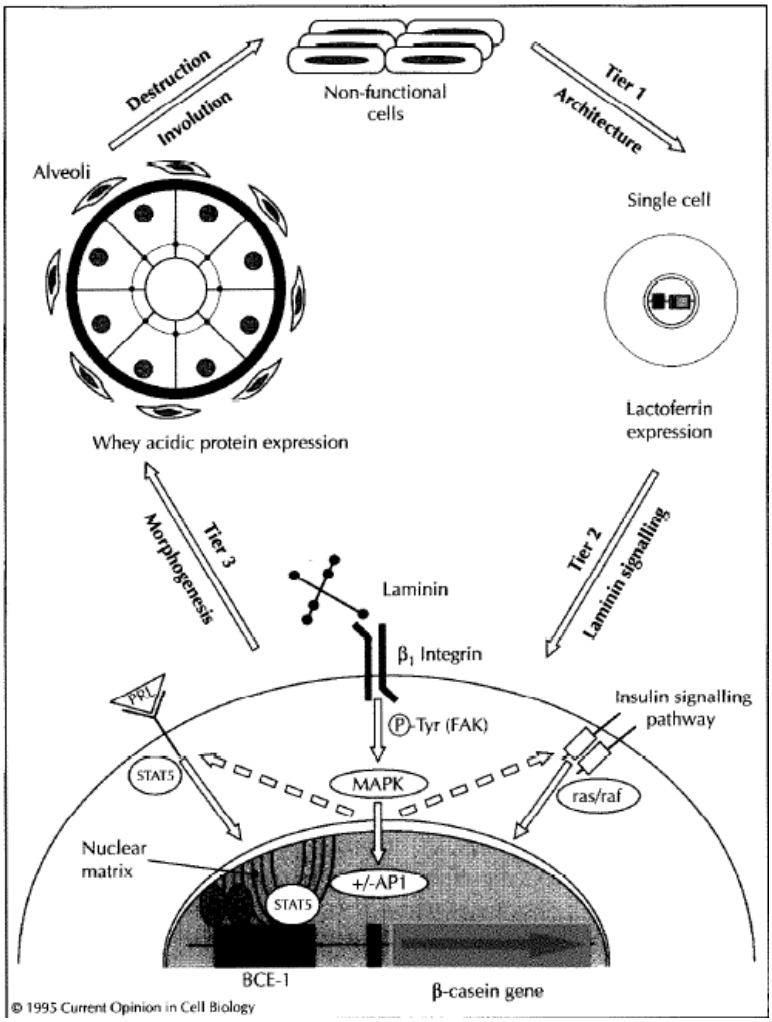
A comprehensive review of the numerous points of intersection between signal transduction pathways initiated by the extracellular matrix, cytokines, growth factors, and differentiation factors.

Figures and Tables

FIGURE 1



A hierarchy of ECM-dependent signals regulates mammary gland development. The first tier of the hierarchy is mediated by architectural changes in cell shape that result in lactoferrin expression. The second tier of the hierarchy is mediated by laminin-specific biochemical signals that activate an ECM-responsive element (BCE-1) and induce endogenous β-casein expression. The third tier in the hierarchy is assembled during morphogenesis and serves to regulate whey acidic protein (WAP) expression and the formation of 'alveoli' in tissue culture models. Destruction of the hierarchy is mediated by matrix metalloproteases and results in involution. Specific signals associated with each tier of the hierarchy are outlined in Table 1. C, CCAATT/enhancer binding protein; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; PRL, prolactin.

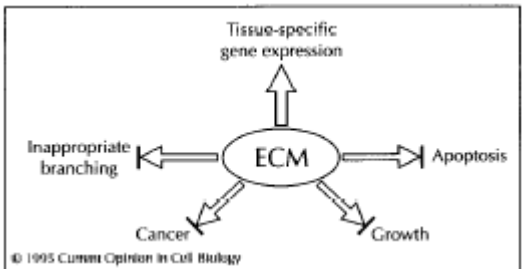
TABLE 1

Table 1. Specific signals associated with each tier of the ECM-dependent hierarchy that regulates mammary gland development.*

Tier	Signals
1. **Architecture**	Decreased adhesion to a rigid substratum Increased cell rounding Reorganization of the cytoskeleton Cessation of proliferation Decreased AP1 transcription factor activity Lactoferrin expression
2. **Laminin signalling**	Laminin-specific β1 integrin clustering and activation FAK phosphorylation, MAP kinase activation and transient increase in AP1 transcription factor activity Modulation of insulin signal transduction pathway required for lactogenic hormone responsiveness Prolactin-dependent activation of STAT-5, which binds to the ECM-response element BCE-1 Proper binding of C/EBP family of transcription factors to BCE-1 Changes in nuclear matrix which affect BCE-1 activity, and require stable integration of the element into the genome Expression of β-casein
3. **Morphogenesis**	Production and deposition of an endogenous basement membrane ECM Downregulation of endogenous TGF-β expression Downregulation of ECM gene expression HGF-induced ductal branching Cell polarization, tight junction formation Vectorial secretion of milk proteins into central lumen Downregulation of endogenous TGF-α expression Expression of whey acidic protein
4. **Destruction/ Involution**	Increased matrix metalloprotease production Decreased production of inhibitors of metalloproteases Basement membrane destruction Entactin fragmentation Increased production and deposition of tenascin Inhibition of milk protein gene expression Interleukin converting enzyme dependent apoptosis Loss of differentiated function

*See also Figure 1. ECM, extracellular matrix; FAK, focal adhesion kinase; HGF, hepatocyte growth factor; MAP, mitogen-activated protein; TGF, transforming growth factor.

FIGURE 2



Extracellular matrix (ECM) as an integrator of function in the mammary gland. ECM-mediated signals positively influence tissue-specific gene expression and inhibit inappropriate branching, growth, apoptosis and the development of cancer.