Chapter 4

THE BIOLOGY OF CONNECTIVE TISSUE: REGULATION OF MESENCHYMAL CELL DIFFERENTIATION BY EXTRACELLULAR MATRIX

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1 INTRODUCTION

As a multicellular organism develops, different cell types express different genes at different times. Although each cell contains the same nuclear information, the uniqueness of each phenotype is the result of molecular controls of gene expression that are exercised at the appropriate place and time in development.

If differentiation is to proceed correctly, the timing of developmental events must be precisely regulated. How this timing occurs within the developing cell remains a major riddle of biology. While it has been appreciated for many years that embryonic cells are able to interpret and act upon signals in their immediate microenvironment, little is known concerning the nature of these extracellular signals or the mechanism of signal transduction within the cell that leads, ultimately, to changes in the pattern of active and inactive genes that characterize developmental phenotypes.

The importance of the extracellular matrix (ECM) in influencing differentiation events has long been known. Pioneering experiments by classical embryologists such as Roux, Spemann, and Holtfreter (reviewed in reference 1) showed clearly that extranuclear as well as extracellular factors provide instructions to the developing cell. With the advent of cell and tissue culture techniques and their application to questions of cellular differentiation, it became apparent that macromolecules in the ECM play an important role in determining which part of a cellular population is to express a particular developmental potential, a process termed embryonic induction. How the ECM alters gene expression in developing cell populations is unknown. We neither know the identity of the biochemical "inducers" in ECM nor are we certain about the mechanisms set in motion by the influence of the inductor on the responding cell type. Yet, by observing ECM-induced developmental

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changes in defined model systems, we are beginning to understand events occurring in reactive tissues that are a consequence of inductive stimuli. The aims of this review are, firstly, to explore how changes in the extracellular matrix signal altered cell behavior so as to influence the genetic potential of the cell, and, secondly, to discuss analogies between in vitro developmental models and cellular differentiation in situ.

II. EXTRACELLULAR MATRIX AND DEVELOPMENTAL INDUCTION

To study gene activation events, facile methods are needed for identifying both developmentally regulated genes and the factors that govern expression of such genes. One model that has been remarkably successful in illustrating specific tissue-matrix interactions in development has been the transformation of embryonic muscle into cartilage in response to bone matrix components. The model has evolved from the observation that allogeneic implantation of rat demineralized extracellular diaphyseal bone matrix into subcutaneous sites results in the local differentiation of endochondral bone (2; reviewed in 3 and 4). A similar differentiation process was observed in vitro when muscle-derived cells formed hyaline cartilage when cultured on a substratum of demineralized bone (5-8). In both cases, the inducing effect was specific for bone matrix and did not occur in response to matrix from tendon, skin, cartilage, or aorta (9). The nature of the inducing signal(s) associated with bone matrix has not been determined but both diffusible (10) and extractable (7) substances have been identified.

In addition to the bone model, numerous other experimental systems have demonstrated the general importance of ECM influences on differentiation (reviewed in 1 and 11-15). In most of these systems, however, understanding of the events that influence gene expression is limited by the complex nature of the differentiation process itself. In developing tissue, differentiation may involve numerous cell populations that differ in their production of, or in their response to, inductive stimuli. This complexity renders extremely difficult the characterization of inductive influences which act on individual cells or on unique cellular populations within the tissue (14). Recently, we described a developmental model that provides a uniform cell population which responds synchronously to an inductive stimulus from the ECM. With this model it has been possible to exploit a unique, developmentally-regulated gene to investigate how ECM modulates the phenotype of a specific cell population from the embryonic stages through terminal differentiation. Although relatively simple, the model presents a useful paradigm of the mechanisms of cell differentiation in more complex tissues and also yields an informational basis for testing additional hypotheses on how other epigenetic requirements, such as cell-cell interactions, growth factors, hormones, etc. (16) influence the differentiation program of a cell.
II. THE LIGAMENTUM NUCHAE AS A DEVELOPMENTAL MODEL

The ligamentum nuchae contains a single fibroblast cell type (17,18) that secretes an extensive ECM of which elastin is the major component. In the ligament of grazing ungulates, such as cows and sheep, elastin comprises greater than 80% of the total tissue protein. Early in the developmental history of this tissue, ligament fibroblasts synthesize predominantly collagen and other matrix proteins but switch to elastin production near the beginning of the last developmental trimester. Biochemical and histological studies (19,20) have documented a period of rapid elastin synthesis that begins near the end of the seventh month of pregnancy (the gestational period for the cow is approximately 270 days) and continues for the first 5-6 months of postnatal life. Thereafter, the rate of increase is much reduced (17,18).

Since a major function of the ligament fibroblast is to synthesize and organize an elastic matrix, elastin production provides a reliable marker of the differentiated phenotype. Other markers of differentiation include the appearance of a β-galactoside-binding lectin on the surface of differentiated cells (Mecham, unpublished results), and the acquisition of chemotactic responsiveness to elastin peptides (21). The cellular simplicity of the bovine ligamentum nuchae, the availability of defined markers of differentiation, and extensive morphological and biochemical descriptions of developmental changes in the tissue make the ligament an ideal model system to address the following questions: 1) How does the ECM influence the selection of options for connective tissue cell development? 2) How is gene expression restricted to appropriate cell types? 3) What are the mechanisms for specific gene regulation during development?

III. DEVELOPMENTAL CLOCK OR EXTRACELLULAR SIGNALS?

The ease with which fetal calf ligament cells can be maintained in culture facilitates testing their developmental potential under controlled conditions. Figure 1 compares developmental changes in the level of elastin synthesis by ligament cells in culture and in minces of fetal ligament tissue. Elastin production increased progressively from low levels in 140-day fetal bovine tissue to a maximum value reached shortly before birth. In the discussion that follows, we utilize the 180-day transition point as a time reference to classify fetal calf ligament cells (abbreviated FCL followed by a number representing the developmental age of the fetus) as either: 1) undifferentiated ("young") cells from fetal tissue less than 180 days of gestation that have not initiated elastin synthesis, or 2) differentiated ("old") cells from fetal tissue older than 180 days that synthesize elastin.

1 In the discussion that follows, we utilize the 180-day transition point as a time reference to classify fetal calf ligament cells (abbreviated FCL followed by a number representing the developmental age of the fetus) as either: 1) undifferentiated ("young") cells from fetal tissue less than 180 days of gestation that have not initiated elastin synthesis, or 2) differentiated ("old") cells from fetal tissue older than 180 days that synthesize elastin.
Figure 1. Changes in the level of elastin production in bovine ligamentum nuchae fibroblasts and in tissue minces during development. Cell cultures and tissue minces were incubated overnight in medium containing β-amino-propionitrile to inhibit crosslinking of tropoelastin. Elastin levels in the medium and cell layer were determined by radioimmunoassay. Values expressed are the mean and standard deviation of duplicate determinations. Adapted from Mecham et al (22).

Total elastin synthesis by cultured cells was less than that of age-matched tissue minces.

The first clue that extracellular signals are required for ligament cell differentiation was evident when FCL-140 cells in long-term culture failed to differentiate spontaneously, even though the culture period included the developmental "window" when cells in intact tissue differentiate and begin elastin synthesis (22). This observation suggested that a developmental clock is not, in itself, directing the transition to elastin production and that an external source of information was needed to integrate the function of cells with the developmental program of the tissue. Extensive studies with glucocorticoids and maternal as well as fetal serum components failed to identify a circulating humoral factor that induced differentiation of the young
ligament cell (23). Consideration of other mechanisms for differentiation brought us to examine the possibility of instructive signals originating from the ECM.

Because differentiation of many connective tissue cells is strongly influenced by cell-matrix interactions (11), we investigated whether ECM from differentiated tissue could induce programmatic changes in undifferentiated cells. To approximate the extracellular matrix of intact tissue, ligament from bovine fetuses of various gestational ages was repeatedly freeze-thawed to kill associated cells and was sliced using a freezing microtome. The tissue slices were then used as a substratum upon which cultured cells were grown. In this way it was possible to expose cells from one gestational age to ECM produced by the same cell type at a different
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stage of development, and thus test the effects on differentiation of matrices that are compositionally and structurally similar to the developing in situ matrix. The results in Figure 2 demonstrate that ECM from fetal ligaments of 270-day gestation triggered differentiation in pre-elastogenic (140-day) cells as assessed by the onset of elastin synthesis. After growing on the tissue matrix for 3 days, 140-day cells synthesized elastin at levels equivalent to cells from fully differentiated tissue. To determine if ligament matrix from pre-differentiated cells could include elastin synthesis, FCL-140 cells were grown on ECM from a 140-day ligament. Figure 2 shows that elastin synthesis by both young and old cells grown on the 140-day matrix was not significantly different from elastin synthesis by cells maintained on tissue culture plastic. Thus, ECM from ligament tissue younger than 180-days (before overt differentiation of ligament cells in situ) had no stimulatory effect on differentiation.

When 140-day cells were separated from inducing matrix by millipore filters or by suspending the tissue matrix on a wire screen above the cells, differentiation did not occur (Figure 3). Likewise, no elastogenic differentiation was observed when undifferentiated cells were seeded at high density onto inducing matrix. However, when these same cells were removed from the matrix with trypsin and reseeded back onto inducing matrix at lower densities that accommodate cell division, elastin production began within 18-24 hours, a period that correlates with the doubling time of the 140-day cell. Thus, the differentiation-promoting effect of the 270-day matrix depends upon cell division after cell-matrix interaction and is not associated with a diffusible substance, since direct cell-matrix contact is necessary to initiate elastin synthesis.

IV. STABILITY OF THE DIFFERENTIATED STATE

The stability of the differentiated state was apparent when matrix-induced cells did not revert to the pre-elastogenic phenotype after removal from inducing ECM. These results, together with the equivalence in elastin production between cultured 270-day cells and matrix-induced 140-day cells, suggest that (a) the total daughter cell population expresses the differentiated phenotype after induction, (b) the entire parent cell population is competent to respond to the matrix inducer, and (c) phenotypic alterations induced by matrix are heritable. Furthermore, once committed to elastin production, the induced cells or their progeny do not require continuous contact with inducing matrix to maintain elastin synthesis. We can conclude, therefore, that the matrix-induced transition to elastin production is a non-reversible conversion to a stable phenotype.

Another important consideration evident from the results depicted in Figure 2 is that, in addition to inducing a permanent phenotypic change in pre-elastogenic cells, ECM plays an important role in maintaining phenotypic stability after differentiation has occurred. FCL-270 cells grown on 270-day ligament matrix produce almost twice as much elastin as cells grown on plastic or on matrix from 140-day ligament. In fact, this higher level approximates closely elastin production by cells in intact tissue and, hence,
Figure 3. Direct cell-matrix contact is necessary for induction of the elastin phenotype. Matrix-induced differentiation of ligament cells does not occur when matrix is separated from cells by a wire screen or by millipore filters.

equalizes the disparity in elastin synthesis observed for cultured cells and intact tissue (Figure 1). It is clear that one of the important functions of ECM is the construction of local cellular environments that will stabilize cell phenotypes and activities. The molecular mechanisms for maintaining phenotypic stability, however, are unknown.
V. BrdU AS A TOOL TO ASSESS GENE ACTIVATION STATES

In many models of differentiation, incorporation of bromodeoxyuridine (BrdU) into DNA inhibits reversibly the development of cell-specific differentiation programs (24). In developing pancreas for example, BrdU alters morphogenesis and reduces the synthesis of pancreas-specific secretory proteins under conditions in which cell proliferation and the rate of total protein synthesis remain normal (25). In the pancreas model it has been well established that BrdU affects a process or processes that are central in controlling cellular differentiation (26).

The precise mode of action of BrdU, which is readily incorporated in place of thymidine into DNA, is not clearly understood. One possible mechanism is that the adduct affects the binding of regulatory proteins to DNA. This has been suggested from several experiments which show that a number of DNA binding proteins have greater affinity for BrdU-substituted DNA (27). BrdU may also alter transcription directly by affecting chromatin structure (28,29). By whatever mechanism, BrdU provides a useful reagent for analyzing the state of specific gene expression during cytodifferentiation. Many genes that are not expressed at a particular stage of differentiation remain inactive (even in the presence of specific inducer stimuli) after BrdU incorporation (24).

When log-phase FCL-130 fibroblasts were cultured with BrdU under conditions in which DNA and total protein synthesis were unaffected, BrdU blocked the induction of the elastin phenotype when the cells were exposed subsequently to inducing matrix (Figure 4). Competency to respond to the matrix inducer was restored, however when BrdU was removed from the medium and replaced with thymidine or when the cells were allowed to divide on the matrix in the absence of BrdU. If undifferentiated FCL fibroblasts were exposed to BrdU after they had contacted the inducing matrix, the transition to elastin synthesis was not affected. Overall, these findings indicate that incorporation of BrdU into the FCL genome before exposure to inducing matrix prevents expression of the differentiated state. In contrast, BrdU has no effect if the cell initiates the new developmental program prior to incorporation of BrdU (22). The requirement for new DNA synthesis and the interruption of differentiation by BrdU provide strong evidence that matrix induction of the elastin phenotype in FCL fibroblasts is a consequence of transcriptional activation of the elastin gene.

VI. THE ELASTIN RECEPTOR AND OTHER CHANGES ASSOCIATED WITH DIFFERENTIATION

Differentiated ligament fibroblasts exhibit directional migration (chemotaxis) to tropoelastin and to peptides derived from cross-linked elastin (30). Undifferentiated cells, however, respond positively to fibronectin and platelet-derived growth factor (PDGF), known chemoattractants for fibroblasts, but do not respond to elastin in the chemotaxis assay (21). Since it is generally accepted that chemotaxis results from ligand binding to specific
Figure 4. The effects of bromodeoxyuridine (BrdU) on induction of bovine elastin synthesis by ECM. A, undifferentiated ligament cells grown for 48 hours on inducing matrix differentiate and begin elastin synthesis. B, matrix-induced differentiation can be blocked, however, if the cells are grown in the presence of BrdU prior to contact with inducing matrix. The inhibitory effects of BrdU could be reversed with the addition of thymidine (B, bottom) or by cell multiplication in the absence of BrdU. C, BrdU has no effect on the differentiation of ligament cells if it is added to the cultures after the cells have contacted inducing matrix.

receptors on the target cell (31,32), these results suggest that undifferentiated cells lack an appropriate "receptor" for elastin.

To determine whether chemotactic responsiveness to elastin, i.e., the appearance of an elastin receptor, appears in coordination with the onset of elastin synthesis, elastogenic differentiation was induced by growing pre-elastogenic cells on inducing ECM. After contact with inducing ECM for 72 hours, the cells demonstrated a chemotactic response to elastin peptides equivalent to fully differentiated cells (Figure 5). As with activation of elastin synthesis, the acquisition of chemotactic responsiveness after ECM exposure required direct cell-matrix interaction and could be blocked reversibly with BrdU (21). Assuming that BrdU prevents the initiation of new
Figure 5. Comparison of chemotaxis to elastin peptides (mean ± SEM, n = 15) of undifferentiated FCL-135 (upper panel) and differentiated FCL-270 (lower panel) cells before and after culture on inducing ECM from a 270-day fetal ligament or on noninducing matrix from a 140-day fetus. Also shown is the chemotactic response of FCL-135 cells cultured in medium preconditioned by inducing matrix. Elastin production is the mean ± standard deviation of triplicate determinations. From Mecham et al (21); reproduced from The Journal of Cell Biology, 1984, Volume 98, pp. 1813-1816, by copyright permission of The Rockefeller University Press.

developmental programs, it would appear that receptor expression requires activation of a specific gene that is not expressed before matrix stimulation. A plasma membrane receptor for elastin has been confirmed recently using classical receptor-ligand binding studies (Wrenn and Mecham, manuscript in preparation).
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In addition to changes in elastin synthesis and the appearance of the elastin receptor, clear differences in the synthesis of other secreted matrix macromolecules characterize ligament cell differentiation. For example, we (33) and Davidson et al. (34) have shown that total collagen production decreases concurrently with the onset of elastin synthesis. In addition, we have described a unique and unusual low molecular weight collagen, FCL-1, the synthesis of which is modulated as a function of gestational age (35) and in response to contact with matrix (36). These metabolic changes, as well as others listed in Table 1, suggest that many genes which define specialized phenotypes are regulated in groups. With the availability of molecular probes for gene activity, it will be of interest to determine whether the elastin gene, the gene for the elastin receptor, and some collagen genes contain common regulatory segments that are under the influence of a master gene that controls the functional phenotype.

TABLE 1. Phenotypic Changes Associated with Ligament Cell Differentiation.

1. Onset of elastin synthesis.
2. Decreased collagen production, including:
   a. type I
   b. type III
   c. FCL-1
3. Inefficient processing of type I procollagen.
4. Appearance or activation of an elastin receptor.
5. Chemotactic responsiveness to elastin peptides.
6. Appearance on the cell surface of a β-galactoside-binding lectin.
VII. THE NATURE OF THE MATRIX INDUCER

It is evident from the results depicted in Figure 2 that only extracellular matrix from late gestation tissue induces differentiation in cultured ligament cells. Despite an extended effort to identify the inducer, the nature of the signal substance that transmits the differentiation signal to the ligament cell is unknown. Yet preliminary results have been helpful in suggesting a direction for characterizing biologically active matrix. Figure 6 shows that ligament matrix does not lose its bioactivity after treatment with agents that extract or denature many connective tissue macromolecules, but the differentiation potential of the ligament matrix is sensitive to treatment with periodate under conditions that are selective for oxidation of carbohydrate moieties. In this respect, ligament matrix induction resembles induction of
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pancreas development by a periodate-sensitive mesenchymal factor (24), except that the ligament factor remains associated tightly with the ECM.

That inducer activity cannot be inactivated or extracted from ligament matrix using high salt concentrations, chaotropic agents, detergents, or reductants emphasizes the difficulty associated with identifying a distinct macromolecular inducer in intact tissue. Our experimental approach to obviate the problem of insolubility was to use ECM from cultured cells as the inducer, since cellular ECM is generally more soluble than tissue matrix. When FCL-120 cells were grown on ECM prepared by lysis of cultured FCL-270 cells, differentiation did not occur. Although this result was contrary to our expectations, it did provide an important insight into the requirements for induction by suggesting that multiple signals are required and that some of these signals originate in early stages of development. The rationale behind this conclusion can best be understood in the context of a model proposed by Caplan et al (37) wherein embryonic development is proposed to proceed through discrete developmental stages, with one cell state (or phenotype) replaced irreversibly by the next more complex state (Figure 7). At each stage of differentiation, the developing cell synthesizes and secretes matrix components that are characteristic of that particular phenotype. As the newly synthesized macromolecules accumulate and interact with preexisting ECM, the cell comes under new environmental influences which induce developmental transitions and, in turn, support the differentiation attained. Thus, in our experiments, undifferentiated cells grown on slices of ligament tissue are exposed to an accumulation of matrix components that represent every stage of cellular differentiation (A through F in Figure 7). As we have shown experimentally, this matrix provides the requisite information for differentiation. In contrast to the composite matrix of tissue, ECM from cultured FCL-270 cells contains only those matrix components that the cell is programmed to synthesize at that particular stage of development (F in Figure 7). The fact that this matrix is not differentiation-competent suggests that critical inductive components are absent from the ECM and are no longer synthesized by the cell. In other words, the differentiated cell in culture does not revert to the earlier phenotypes that would be necessary to restructure a tissue-like matrix. This reflects the fact, discussed earlier, that the determined state is relatively stable and suggests that the ligament cell is assigned its destiny in a series of irreversible steps guided by influences from the ECM. The nature of the signal for differentiation at each step is unknown but because there is only one cell type in the ligament, differentiation must be occurring through a series of feed-back influences from an ECM that can only be provided by the ligament cell population itself. In this model, the effector cell is also the target cell.

The scheme proposed in Figure 7 postulates a gradual restriction of developmental options available to the developing cell and incorporates what Saxen et al (38) have defined as permissive and directive influences. Under a directive influence a multipotent cell possessing more than one developmental option becomes committed to one specific pathway. A permissive influence or condition enables the responding cell to express functionally a pre-existing differentiative bias. Thus, cells must have received directive messages before permissive signals can activate the appropriate phenotype. As differentiation progresses, the developmental options available to the cell
Figure 7. Proposed model of ligament cell differentiation. Ligament cell development occurs as a series of highly restricted phenotypic changes that are the result of the cellular genetic program and inductive influences from the microenvironment. The phenotype at each differentiation state (A through G) defines the synthesis of specific matrix components that accumulate extracellularly and, in turn, feed back to influence progressive differentiation. ECM from bovine tissue younger than 180 days (A through D) cannot induce elastin synthesis in pre-elastogenic cells. By 270 days (A through F), however, ECM becomes induction-competent. ECM from 270-day cells (F only) has no effect on elastogenic differentiation. The suggestion, then, is that initiation of the developmental program requires the complete matrix, A-F.

become restricted so that signals change from predominantly directive to mostly permissive. While this model of differentiation is undoubtedly oversimplified, many of its assumptions are testable using the ligament model. Since elastin production begins relatively late in development, it is likely that matrix-induced stimulation of elastin synthesis is in response to a permissive stimulus. This assumption is supported by the finding that ligament cells from fetuses younger than 90 days gestation fail to differentiate in response to inducing matrix (Mecham et al., manuscript in preparation). We interpret this result to indicate that cells younger than 100 days are not competent to respond to the matrix inducer and that an instructive event occurs at around gestation day 100 that enables the ligament cell population to recognize differentiation signals. Once again the sequential nature of gene control is obvious: ligament cells are capable of
responding to matrix induction only during a finite (and appropriate) period of their development.

VIII. SPECIFICITY OF INDUCTION

The above discussion raises the question of whether matrix-induced differentiation is ligament-specific or represents a more general phenomenon functional for all elastogenic as well as non-elastogenic cell types. By way of approaching this issue, inquiries amenable to experimental study suggest themselves. First, is the pre-elastogenic ligament fibroblast simply a "primed" cell that will respond to any matrix signal, or is ligament matrix required exclusively for differentiation? To answer this question, the effects of non-ligament and ligament ECM on induction of elastin synthesis were compared by culturing undifferentiated ligament cells on bovine amnion or on 270-day ligament slices (Mecham et al., manuscript submitted for publication). The amnion is avascular and contains three basic tissue components: epithelium, basement membrane, and collagenous stroma. The epithelium can be removed easily with dilute detergents, leaving a surface of intact basement membrane containing collagen types IV, V, and laminin (39). Denuded amnion thus provides two surfaces of ECM to test for induction effects: the stromal surface containing interstitial collagen types similar to those found in fetal ligament, and the basement membrane side containing matrix components not normally encountered by a fibroblast. When these matrices were tested in the induction assay, FCL-120 cells did not differentiate after contact with either side of the amnion. This and results from other studies suggest that a ligament-specific interaction is essential for ligament cell differentiation; the signal for induction does not reside in non-ligament ECM.

At this point, the question of whether or not non-ligament, pre-elastogenic cells can be induced to make elastin by ligament ECM is a crucial one. Is the instructive signal that is associated with 270-day ligament matrix recognized by all cells or only by ligament cells? To investigate the specificity of matrix induction, skin cells and chondroblasts from hyaline cartilage from a 140-day bovine fetus were cultured on 270-day ligament matrix. No elastin production could be detected in either cell type under conditions that induced elastin synthesis in ligament fibroblasts from the same fetus. The failure of skin cells to respond to the ligament matrix is important since elastin production in skin and ligament begin at about the same time during embryogenesis. Clearly, skin cells require a different signal to initiate elastin synthesis.

We can conclude from these experiments that there is tissue specificity inherent in matrix induction of ligament cell differentiation: ligament cells respond to ligament matrix only, and ligament matrix does not induce elastin production in non-ligament cells, including cells in other tissues that are programmed to synthesize elastin. The implication, then, is that the specificity of induction lies with both the cell and the ECM: there is a requirement for proper signal recognition by the cell (the appropriate "receptors," perhaps) and the presence in the ECM of the correct signals (Figure 8).
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Figure 8. Models explaining species specificity in matrix induction. Non-ligament cells in contact with ligament matrix (left) are not induced to make elastin because they lack the appropriate receptors for detecting instructive signals associated with the ligament ECM. In contrast (right), pre-elastogenic ligament cells, which can recognize and react to ligament ECM, do not differentiate when cultured on non-ligament matrix due to the absence of appropriate inducers in the non-ligament tissue.

IX. MECHANISM OF MATRIX INDUCTION

Matrix-induced differentiation is an adaptation by cells to a changing environment (13). In the case of the developing ligament, the environmental changes are controlled largely by the ligament cell itself. As has been shown above, induction and control of the differentiated state is not simply a function of the ECM, but must also involve the expression of specific recognition macromolecules on the cell surface. Accordingly, mechanisms which could account for matrix induction must be considered in this context.

Figure 9 illustrates two possible mechanisms for signalling between cell and matrix. Although these examples are oversimplified, they can be useful in a pedagogic sense to illustrate the complexities involved in how a cell communicates with its microenvironment. In the first instance, the correct combination or concentration of inducer macromolecules appears in the extracellular matrix at an appropriate time during development. These molecules are recognized by existing receptors on the plasma membrane and differentiation occurs. In the second model, developing cells become sensitive to existing extracellular signals by activating new receptors or by modifying internal signals produced in response to the inducer. An important distinction between these two mechanisms relates to time and how cells sense time. In biological systems time has its basis in rate constants (see reference 37 for a more thorough discussion of this topic). If the primary signal for differentiation resides with the macromolecules of the ECM then
Figure 9. Possible mechanisms for matrix-induced differentiation. Top: the correct combination of inducer components appears in the ECM at the appropriate time during development and is recognized by existing receptors on the plasma membrane of a primed cell. Bottom: Developing cells become sensitive to existing ECM signals by activating new receptors or by modifying internal signals produced in response to the inducers.

Timing of differentiation events depends upon the stability (decay balanced by synthesis) of matrix components. However, with the expression of new receptors, timing mechanisms can be built into the genome and regulated by a molecular clock. This explanation offers the advantage of allowing receptor expression to be transient and to represent a more finely regulated "time-dependent" component of differentiation than is afforded by changing the ECM. The precise mechanism used by the cell to channel differentiation is undoubtedly a combination of the two possibilities depicted in Figure 9 and it is impossible at present to identify a particular pathway associated with a specific differentiation event. Nevertheless, it is important to realize that such events do occur in biological systems.
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How differentiation signals are transmitted from the ECM to the nucleus is unknown. In the ligament model, steroid hormones (23) and cyclic nucleotides (41) play no direct role in this process. The apparent regulatory complexity described throughout this chapter suggests that matrix-induced differentiation must be controlled by multiple mechanisms. It is possible that differentiation is regulated covertly by way of cues from accumulated endogenous or exogenous inducers (42), or by the dilution of inhibitors (43,44). Alternatively, differentiation might be signaled by changes in composition of the ECM resulting from alterations in the relative proportions of individual matrix components as a consequence of variations in synthetic or degradative rates. Changing combinations of connective tissue components and the resultant rearrangement of ECM may provide structural signals that direct differentiation via an ECM-dependent reorganization of the cytoskeleton (45,46). It is significant in this regard that when young ligament cells are induced by ECM to synthesize elastin their morphology changes from bipolar to stellate, thereby resembling late gestation, fully differentiated cells. Similarly, late gestation cells become increasingly bipolar as they lose the elastin phenotype in response to repetitive subculture. Whether or not a particular cell shape is a reflection of the differentiated state, the cell surface and the receptors that link the ECM with the cytoskeleton must be considered as potentially important modulators of gene expression (47).

X. MATURATION AND TERMINAL DIFFERENTIATION: THE ADULT PHENOTYPE

The study of developmental events in mammalian erythroid cells has distinguished three main stages in their differentiation. The first stage is characterized by the transition from "stem" cells to cells "committed" to differentiate into a single cell type. During the third stage, which is referred to as terminal differentiation, differentiated cells mature and become specialized in function, and this process includes changes in cell proliferation potential (48). Although the erythroid cell may not be an appropriate analogy for tissue-associated cell types (49), within the general pattern of the differentiative process all three stages apply to ligament cell development.

We have already discussed the commitment and differentiation phase of ligament development. The existence of a "maturation" phase is suggested by age-associated changes observed in biochemical and histological studies (18,20). A progressive decrease in cell density begins with the onset of elastin synthesis (differentiation) and continues until a constant level is reached by about the third month postnatally. At this time, ligament cells in vivo assume a morphology that is characteristic of the resting form of the adult ligament cell and the rate of increase of elastin deposition falls off dramatically. A clearer definition of the end-stage phenotype is evident, however, when cells from adult tissue are studied in vitro. The primary isolation of cells from adult ligament tissue yields a fibroblast cell type that differs both functionally and morphologically from the fetal cell (Mecham et al, manuscript in preparation). The adult cell in culture spreads over a larger surface area than fetal cells and contains numerous phase-dense stress fibers.
Figure 10. Comparison of elastin synthesis by adult ligament cells (ALN) and fetal cells from a 120-day and 270-day fetus. When grown on plastic, adult cells synthesize elastin at a rate between that of the late and early fetal cells. Elastin synthesis is stimulated in all three cell types after contact with inducing ECM.

Cultures of adult cells show true contact inhibition whereas fetal cells in vitro continue to proliferate after confluence to form multilayers. In addition, a comparison of culture growth kinetics showed that the adult cells have a population doubling time that is more than twice that of the fetal cells (72 hours for adult cells compared to 22 hours and 18 hours for FCL-270 and FCL-110 day cells, respectively).

Although adult cells in culture synthesize elastin, they do so at a rate that is about 50% less than that of FCL-270 cells (Figure 10). In chemotaxis assays, adult cells show a positive chemotactic response to elastin peptides, suggesting that they possess a functional elastin receptor. To study the reversibility of the adult phenotype, we grew adult cells on matrix slices from 270-day ligament tissue. Figure 10 shows that exposure to ECM stimulated elastin production more than two-fold in the adult cells with no concomitant change in the cellular doubling time. Unlike phenotypic induction of early fetal cells by ECM, matrix stimulation of adult cells was not blocked.
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by BrdU and elastin production reverted to low levels when the adult cells were removed from the matrix substratum. On the basis of these observations we can conclude that the elastin gene is operative but expressed at a low level in the adult cell. Yet, under appropriate conditions, elastin synthesis can be reversibly stimulated. It will be of interest to know under what circumstances and in response to which stimuli the adult cells become reactivated in the tissue.

XL CONCLUSIONS

Figure 11 depicts a chronology of elastin phenotype development in the bovine ligamentum nuchae. Our studies have distinguished four main stages in the differentiation of the ligament fibroblast. The first stage, which begins shortly after the first developmental trimester (starting at A in Figure 11), is characterized by the transition of a mesenchymal precursor cell to a cell "determined" or "committed" to the elastogenic pathway. The determined state is stable and does not depend on a constant environment for its integrity. Processes involved in cellular commitment are unknown as is the nature of the influences that might direct this process. Committed cells have developed an elastogenic bias and are primed to differentiate, or express the final functional phenotype, upon exposure to an appropriate exogenous stimulus. Determination and differentiation may be closely coupled in many tissues, but for ligamentum nuchae this does not appear to be the case: cells as young as 100 days of gestation differentiate to produce elastin when exposed to an inducing matrix. The separation in time between commitment and differentiation illustrates the independence of the two events.

![Diagram](image)

**Figure 11.** The comparative times of restrictive and expressive events in ligamentum nuchae development. Differentiation, which begins at around 180 days, is assessed by the activation of the elastin phenotype.
Differentiation (stage B) of the ligament cell is initiated, either fully or in part, by signals associated with the extracellular matrix. It is not yet known whether the ECM plays an instructive or permissive role nor is it known how the cell recognizes and interprets the differentiation signal. Once the differentiation program has begun, however, the cell no longer requires direct contact with the matrix inducer in order to express the elastin phenotype. The molecular changes that characterize the elastin phenotype are stable and heritable and remain active through the period of elastin deposition in the tissues until elastogenic cells enter a period of relative inactivity (stage C) characterized by decreased elastin and total protein synthesis and a loss of proliferative potential. The function of these end-stage cells is unknown but their phenotype may be adapted to play a general role in repairing tissue damage through processes such as wound healing (stage D). The kinds or the extent of reactivating signals has not yet been investigated but will obviously be important in understanding diseases involving elastin-containing tissues.

Many questions about matrix effects on determination and differentiation remain to be answered. For example, do instructive influences from the cellular microenvironment directly differentiate as a cascade of binary choices between alternative states of determination, with each decision being separated by at least one round of cell division, or are cells able to make multiple, rather than binary choices between many different states? The ligamentum nuchae provides an ideal model to investigate such questions. The fairly rapid and synchronous differentiation of ligament cells in response to inducing signals has opened up the possibility of exploiting molecular biology to study how the cell controls genetic material during development.

REFERENCES


