Regulation of α1(I)-Collagen Gene Expression in Response to Cell Adhesion in Swiss 3T3 Fibroblasts*

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Nonadhesive conditions cause Swiss 3T3 fibroblasts to enter a quiescent state that is reversed upon reattachment to a surface. Previously, we demonstrated that adhesion in serum-free conditions is sufficient to activate suspension-arrested cells out of G0, with the induction of the growth-associated genes, c-fos, c-myc, and actin.

In this study, we have employed this system to identify programs of gene expression that respond primarily to the adhesive state of the cell, rather than the growth state. We show that cells in different adhesive states can be distinguished by their patterns of protein synthesis. Analysis of one adhesion-responsive protein led to its identification as pro-α1(I)-collagen. Pro-α1(I)-collagen synthesis and mRNA levels are decreased up to 6-fold in suspension-arrested fibroblasts, but are enhanced up to 5-fold as cells approach confluence. This suggests that the reduced expression in suspension-arrested cells is not simply a result of quiescence. In addition, reattachment of suspended cells in serum-free conditions caused a 7-fold induction of collagen mRNA levels and a >20-fold rise in the rate of procollagen synthesis. The expression of c-myc was induced during adhesion in serum-free medium as well as by serum addition to suspension-arrested cells. However, α1(I)-collagen gene expression was unaffected by serum in the absence of adhesion. These results indicate that collagen gene expression is directly responsive to cell adhesion, independent of the growth state.

The interactions of cells with the extracellular matrix (ECM) or with other cell types play an important role in growth and morphogenesis during early development and in wound healing, tissue repair, and regeneration in the adult organism (Grobstein, 1975; Kleinman et al., 1981; Hay, 1981; Treistad, 1984; Ekbom et al., 1986). Several cell culture systems have been developed to understand these multiple functions of the ECM in growth and differentiation. They include mesenchyme (Tomasek and Hay, 1984), mammary epithelium (Li et al., 1987; Blum et al., 1987), adipocytes (Spiegelman and Ginty, 1983), synovial fibroblasts (Aggeler et al., 1984), chondrocytes (Zanetti and Solursh, 1984), ovarian granulosa cells (Ben-Ze'ev and Amsterdam, 1987), hepatocytes (Bissell et al., 1987; Schuetz et al., 1988; Ben-Ze'ev et al., 1988), and endothelial cells (Inger et al., 1987; Grant et al., 1989). In some of these systems, the expression of tissue-specific genes is dependent upon adhesion to particular ECM components.

Cell-ECM interactions change continuously during growth and development as a result of cell migration, but also as a result of changes in the expression of the ECM components and their receptors. It has been postulated that this change in expression of cell adhesion components is itself regulated by specific cell-ECM interactions and that such expression can influence the phenotype of the cell (Bissell et al., 1982; Farmer and Dike, 1989; Inger and Folkman, 1989). However, a direct relationship between expression of cell adhesion proteins and the adhesive state of the cell has been difficult to establish due to multiple variables in the culture systems. For instance, in studies where the synthesis of actin (Farmer et al., 1983) and vinculin (Ungar et al., 1986; Bendori et al., 1987) appear to be regulated by the extent of cell-cell and/or cell-matrix contact, the experimental conditions (high cell density and suspension culture) also affected the growth state. In addition, soluble factors such as transforming growth factor-β and epidermal growth factor that regulate cell proliferation also regulate the expression of actin (Elder et al., 1984; Leof et al., 1986), collagen and fibronectin (Ignutz and Massague, 1986; Ignutz et al., 1987; Blatti et al., 1988), and integrin (Ignutz and Massague, 1987).

We have developed a cell culture system that permits an analysis of the effect of cell adhesion in the absence of growth factors or other soluble serum factors. The system involves suspension of cells in methylcellulose followed by adhesion to a solid substrate. Earlier studies by Pennman and co-workers (Benecke et al., 1978, 1980; Farmer et al., 1978) revealed that suspension culture of mouse fibroblasts had a dramatic effect on overall macromolecular metabolism. We further demonstrated that prolonged suspension of an exponentially growing population of cells leads to arrest in G0. Subsequent adhesion in the presence of serum activated re-entry into the cell cycle, with events commonly associated with the G0/G1 transition (induction of c-fos, actin, and c-myc mRNAs) followed by progression into S phase (Dike and Farmer, 1988). Activation of these suspension-arrested cells out of G0 did not depend entirely on availability of serum factors since adhesion in the absence of serum induced c-fos and c-myc mRNA. However, progression to DNA synthesis required growth factors.

In the present study, we have employed this system to identify programs of gene expression that respond primarily to the adhesive state of the cell rather than to the growth state. We demonstrate that α1(I) collagen mRNA levels and pro-α1(I)-collagen synthesis are depressed in suspended fibroblasts, whereas density-arrested cells show high levels of
collagen gene expression. Thus, reduced collagen expression in the absence of adhesion is not simply a consequence of growth arrest. Activation of these cells by addition of serum to suspension cultures can induce c-myc. However, activation of suspension-arrested cells by reattachment in the presence or absence of serum induced collagen gene expression severalfold. These results show that Type I collagen gene expression responds directly to the adhesion state of the cell, independent of its growth state.

MATERIALS AND METHODS

Cell Culture—Early passage Swiss 3T3 mouse fibroblasts were obtained from Dr. J. N. Chou, Dept. of Microbiology, Boston University School of Medicine. Cells were grown in DMEM (GIBCO) supplemented with glutamine, penicillin/streptomycin, and 10% calf serum (HyClone) in a 10% CO₂ atmosphere. Cultures were started from frozen stocks and passed no more than five times. For suspension experiments, cells were plated in 150-mm Falcon tissue culture dishes and grown to ~70% confluency. Cells were toyopoxized, dispersed in serum-containing medium, and added as a single cell suspension to a final density of 2 × 10⁵ cells per ml to Nalgene bottles which contained 1.5% Methocel (4000 centipoise, Dow Chemicals) in DMEM/10% serum. After dispersion in Methocel, an aliquot was removed for microscopic examination to ensure a single cell suspension. Cells were recovered from Methocel by dilution with room temperature PBS and centrifugation. Cells were density-arrested by growth to confluence after feeding at 70% confluency.

Analysis of Newly Synthesized Proteins—Adherent cells were labeled with 100 µCi of Tran³S label (ICN) per ml of methionine-free or methionine- and cysteine-free media. Cells were recovered from Methocel by dilution with room temperature PBS and centrifugation. Cells were density-arrested by growth to confluence after feeding at 70% confluency.

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RESULTS

Patterns of Protein Synthesis Distinguish Cells in Different Adhesive States—Suspension of 3T3 fibroblasts in methylcellulose leads to growth arrest in G₀ even in the presence of serum. Comparison of suspension-arrested cells with density-arrested monolayer cells might therefore reveal features specific to the lack of adhesion rather than the growth arrest, because the only variable between these two culture conditions is the degree of cell adhesion. To assess the effects of abolition of cell adhesion on the overall pattern of gene expression, we analyzed newly synthesized proteins by high resolution two-dimensional gel electrophoresis. Each adhesive state (Fig. 1: subconfluent (A), suspended (B), and confluent (C)) shows a characteristic pattern of protein synthesis. It is apparent that many proteins are regulated in a manner that depends on the growth state (compare Fig. 1A with Fig. 1B and C). However, there are proteins whose synthesis appears to be regulated by changes in adhesion. Specifically, a group of high molecular weight proteins (100,000 > 200,000, apparent pl 6.0–7.0), whose synthesis decreases dramatically in suspension culture, and two groups of lower molecular weight proteins (60,000–65,000, pl ~5.0; and 35,000–50,000, pl 7.5–8.0) whose synthesis is induced in response to lack of adhesion. We have focused these studies on a ~150-kDa protein (labeled C in Fig. 1A) synthesized at relatively high rate in density-arrested cells (Fig. 1C), and at barely detectable rates in suspended cells (Fig. 1B).

Identification of the 150-kDa Protein (C) as a Collagen—The synthesis of protein "C" was induced by treatment of suspension-arrested cells with transforming growth factor-β, a well-characterized regulator of ECM production. In addition, a protein of similar electrophoretic properties was found to be a major component of the conditioned medium of density-arrested cells (data not shown). These data are consistent with the possibility that this protein is an ECM molecule, perhaps a collagen. Accordingly, we labeled cells with [³⁵S]proline, given the known abundance of prolyl residues in the triple helical regions of collagen. A comparison of two-dimensional gels of total [³⁵S]methionine-labeled cell layer proteins with total [³⁵S]proline-labeled extracts (Fig. 2A) revealed that the major [³⁵S]proline-labeled 150-kDa species co-migrated with the [³⁵S]methionine-labeled protein C. To definitively identify this protein as a collagen, we digested total cell proteins, isolated from cells labeled for 1 h with [³⁵S]methionine, with highly purified bacterial collagenase. Two-dimensional gel analysis (Fig. 2B) showed that the 150-kDa proline-rich species is collagenase-sensitive. Collagenase digestion of [³⁵S]labeled secreted proteins followed by two-dimensional PAGE also resulted in the selective loss of the same species,
Type I Procollagen mRNA Levels Are Regulated by Cell Adhesion—Previous studies have shown that suspension of mouse fibroblasts results in a dramatic reduction in overall protein synthesis that can result from changes at both the translational and transcriptional level (Benecke et al., 1980; Ben-Ze’ev et al., 1980). To determine the level at which collagen gene expression was regulated by suspension, we analyzed steady state levels of mRNA. Northern blot analysis of total RNA (Fig. 3) from growing cells and cells entering suspension or density arrest revealed that α1(I)-collagen mRNA levels drop 3–6-fold in suspension (Fig. 3, lanes 2–4) and are substantially increased (3–5-fold) in density-arrested cells (Fig. 3, lanes 5–7). Thus, α1(I)-collagen mRNA levels changed similarly to the synthesis of the putative procollagen (protein species C in Fig. 1). The expression of fibronectin, another component of the ECM of fibroblasts, did not follow this pattern. Fibronectin mRNA levels rose 3–5-fold by 24 h in suspension and remained at this level. Density-arrested cells showed low levels of fibronectin mRNA. Thus, although both ECM-encoding mRNAs are adhesion-responsive, they are regulated differently.

It might also be expected that the mRNAs encoding major components of the cytoskeleton would respond to the alteration of the adhesive state. However, steady state levels of cytoskeletal actin and β-tubulin mRNAs decreased 3–5-fold in both suspended and density-arrested cells. Vimentin mRNA levels remained fairly constant throughout the times analyzed in both systems. Thus for these genes, it was not possible to separate their response to lack of adhesion from their response to a change in growth state. Histone 3.2 mRNA levels were monitored as an indicator of the growth state. Expression of this S-phase specific gene was suppressed as cells entered quiescence in suspension and as a confluent monolayer of cells. DNA synthesis was measured by autoradiography of nuclei labeled during a 2-h pulse with [3H] thymidine. In both suspension and density-arrested cells, less
immediately at the same density or were suspended in Methocel for 24 h (lane 2), 48 h (lane 3), or 72 h (lane 4); confluent cells at 24 h (lane 5), 48 h (lane 6), and 72 h (lane 7) after medium renewal. Filters were exposed to Kodak x-ray film for 5 h (collagen, actin, vimentin) or 16 h (fibronectin, tubulin, histone), with Cronex lightning plus intensifying screens.

FIG. 4. Transient detachment and serum do not affect ECM gene expression during suspension culture. Growing subconfluent cells (lane 1) were trypsinized and replated at the same density for 6 h (lane 2) or 18 h (lane 3). Cells were also suspended in 0.4% serum + 250 µg/ml soybean trypsin inhibitor for 2, 6, or 18 h (lanes 4-6, respectively) or in 10% serum for 2, 6, or 18 h (lanes 7-9, respectively). Total RNA (7 µg/lane) was isolated from each of these conditions and subjected to Northern analysis.

FIG. 3. ECM mRNAs are differentially regulated in suspended and confluent cells. Northern analysis of total RNA (10 µg/lane) from asynchronous, subconfluent cells (lane 1); cells suspended in methocel for 24 h (lane 2), 48 h (lane 3), 72 h (lane 4); confluent cells 24 h (lane 5), 48 h (lane 6), and 72 h (lane 7) after medium renewal. Filters were exposed to Kodak x-ray film for 5 h (collagen, actin, vimentin) or 16 h (fibronectin, tubulin, histone), with Cronex lightning plus intensifying screens.

The process of suspending cells requires trypsinization and addition of fresh serum. This raises the possibility that the change in collagen gene expression is a direct response to removal of cell-derived matrix and/or addition of serum. To address these possibilities, cells were trypsinized and replated immediately at the same density or were suspended in Methocel. Northern analysis (Fig. 4) showed that while suspension led to a rapid reduction of collagen mRNA levels, transient detachment from a surface was not sufficient to bring about the changes seen after prolonged suspension. Serum deprivation of smooth muscle cells has been shown to enhance collagen gene expression (Kindy et al., 1988). If serum factors were responsible for the decrease in collagen gene expression in suspended cells, one might expect a rise in collagen mRNA on removal of serum. However, in 3T3 cells, serum deprivation did not reverse the decline in α1(I)-collagen mRNA. Suspension of cells in the absence of serum, (using soybean trypsin inhibitor to inactivate trypsin) led to the same down-regulation of collagen mRNA levels as in the presence of serum (Fig. 4, compares lanes 4–5 with 7–9). Thus, the adhesion-dependent regulation of collagen mRNA levels overrides any serum effects. Fibronectin mRNA levels were unchanged or rose very slightly in response to these manipulations at the time points tested.

Reattachment of suspended cells in the presence or absence of serum resulted in a dramatic increase in collagen mRNA levels (Fig. 5). Northern blot analysis of RNA from cells replated for 2, 6, and 18 h on tissue culture plastic in either 0.4 or 10% serum (Fig. 5) showed that collagen mRNA levels increased 7-fold by 18 h after reattachment. The kinetics of the maximal adhesion response of this gene, however, were delayed in the presence of serum (Fig. 5, compare lanes 6–8 with 9–11). Previous studies (Dike and Farmer, 1988) have revealed that adhesion of suspension-arrested cells results in the G0-G1 transition, as evidenced by the sequential induction of c-fos, actin, and c-myc mRNAs. It was conceivable, therefore, that the induction of collagen mRNA was linked to this programmed transition out of the suspension-arrested state. We have found that c-fos and c-myc gene expression can be induced in the absence of adhesion by the addition of purified growth factors or serum to suspended cells.3 Comparison of gene expression activated by these different means, i.e. serum stimulation in suspension versus adhesion to a surface, is also shown in Fig. 5. Addition of serum to suspension-arrested cells (Fig. 5, lanes 3–5) induced c-myc expression, but had no effect on collagen mRNA levels. Similarly, serum stimulation of density-arrested cells (Fig. 5, lanes 13–15) had no effect on the high levels of collagen mRNA during the time analyzed. However, serum did induce fibronectin mRNA in density-arrested cells, along with c-myc, consistent with the data demonstrating that fibronectin is a serum-responsive gene (Blatti et al., 1988).

Cell Adhesion Regulates Synthesis of Type I Procollagen—To assess whether the increase in collagen mRNA resulted in a corresponding increase in procollagen synthesis, reattaching cells were pulse-labeled with [35S]methionine or a mixture of [3H]- and [14C]proline. Newly synthesized proteins were ana-

Type I procollagen synthesis is induced by reattachment of suspension-arrested 3T3 cells. Suspended cells were allowed to reattach in the presence or absence of serum. Duplicate dishes containing equal numbers of cells (2 × 10⁸/p60) were labeled with 20 μCi/ml of [¹⁴C]proline (Du Pont-New England Nuclear) + 50 μCi/ml of [³H]proline (Amersham) or 100 μCi/ml of Tran³⁵S label (ICN) for 1 h. Total cell-associated proteins were extracted into lysis buffer A. A, amounts of [¹⁴C]proline-labeled extracts normalized to equivalent counts/min of [³H]proline-labeled proteins isolated from cells suspended for 2, 6, or 18 h; lanes 6–8, suspended cells reattached in 10% serum for 2, 6, or 18 h; lanes 9, markers. B, two-dimensional gel electrophoresis of equal amounts of [³H]proline-labeled extracts normalized to equivalent counts/min of [³⁵S]-labeled proteins isolated from cells suspended for 48 h (lane 1), or allowed to adhere to tissue culture plastic in the absence of serum for 2 (lane 2) and 6 (lane 3) h.

**FIG. 6.** Type I procollagen synthesis is induced by reattachment of suspension-arrested 3T3 cells. Suspended cells were allowed to reattach in the presence or absence of serum. Duplicate dishes containing equal numbers of cells (2 × 10⁸/p60) were labeled with 20 μCi/ml of [¹⁴C]proline (Du Pont-New England Nuclear) + 50 μCi/ml of [³H]proline (Amersham) or 100 μCi/ml of Tran³⁵S label (ICN) for 1 h. Total cell-associated proteins were extracted into lysis buffer A. A, amounts of [¹⁴C]proline-labeled extracts normalized to equivalent counts/min of [³H]proline-labeled proteins isolated from cells suspended for 2, 6, or 18 h; lanes 6–8, suspended cells reattached in 10% serum for 2, 6, or 18 h; lanes 9, markers. B, two-dimensional gel electrophoresis of equal amounts of [³H]proline-labeled extracts normalized to equivalent counts/min of [³⁵S]-labeled proteins isolated from cells suspended for 48 h (lane 1), or allowed to adhere to tissue culture plastic in the absence of serum for 2 (lane 2) and 6 (lane 3) h.

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**DISCUSSION**

The long term aim of these investigations is to identify genes that are expressed in direct response to the interaction of extracellular matrix with cell surface receptors and to define the molecular mechanisms that regulate these genes. Such an endeavor is dependent on a cell culture system that can distinguish between the response of cells to several different effectors; in particular, those associated with adhesion to ECM proteins and those associated with binding of soluble growth factors. In the present study, we demonstrate that there are significant changes in overall gene expression which appear to correlate with the adhesive state of the cell rather than the growth state. Regulation of pro-α(I)-collagen gene expression is demonstrably anchorage dependent, while that of other ECM and cytoskeleton proteins such as fibronectin, vinculin, and actin appears to be responsive to both the growth and adhesion state of the cell. Collagen gene expression is increased in a manner that is consistent with the formation of cell-matrix interactions, i.e. reattachment of suspended cells and increased density of adherent cells (production of ECM is known to be enhanced in confluent cells).

Collagens are known to be rather basic proteins with pI values reported in the range of 8-9 (Little and Church, 1978). Therefore, the identification of a protein (C) with characteristics of a procollagen such as molecular weight, proline richness, and collagenase sensitivity but an apparent pI of 6.5-7 requires some consideration. Estimation of the pI values of purified rat type I collagen chains from their position on two-dimensional gels, yields values of 8.7 and 9.2 for the α₁(I) and α₂(I) chains, respectively (Tanaka et al., 1981). However, mature collagen chains are generated by cleavage of the acidic propeptides from procollagen molecules. Our studies show that collagenase treatment of biosynthetically labeled cell-associated and secreted proteins generates two 25-30-kDa peptides of pI 5.4 and 5.2, concomitant with the obliteration of the 150-kDa putative procollagen. (Bacterial collagenase cleaves the X-Gly bond contained in Pro-X-Gly-Pro sequence in the triple helical region of collagens.) Thus, it is possible that the presence of the propeptides as well as post-translational modifications contribute to the focusing of the mouse α₁(I)-procollagen chain in the observed range of 6.5-7.0. However, the pro-α₂(I) chain which is detectable in SDS-PAGE of proline- or methionine-labeled extracts is probably still too basic to resolve in our two-dimensional PAGE system.

Previous studies have suggested that the mRNA levels of interstitial collagens, both types I and III, are apparently highly responsive to the growth state of cells. An increase in type I and III mRNA levels is observed when growth of either fetal lung fibroblasts (Misculin et al., 1986) or smooth muscle cells [SMC] (Steppe et al., 1986; Liau and Chan, 1989) is arrested at high cell density. Our studies strongly indicate that the regulation of type I collagen is not related directly to entry of cells into a quiescent state, but is related to the change in cell morphology accompanying the growth arrest. Fibroblasts maintained in suspension culture for prolonged periods of time also enter a quiescent phase. However, collagen mRNA levels are regulated in a manner that is consistent with changes in cell adhesion. This observation may also explain the contradictory results on the expression of type I collagen during growth arrest induced by serum deprivation in smooth muscle cells. Sonenshein and co-workers (Kindy et al., 1986; Liau and Chan, 1989) demonstrated that only type III collagen was serum-responsive in rabbit SMC. It is possible that these individual SMC cultures differ in the degree of cell-matrix interactions. This property of the system may therefore be the primary regulator of type I collagen gene expression rather than the serum.
Furthermore, it appears from the present studies that serum is able to modulate the expression of collagen in a manner that is not simply related to growth state. In reattaching cells, serum decreased the extent of adhesion-activated collagen expression. Yet, addition of serum to cells maintained in suspension does not further down-regulate collagen mRNA levels. This suggests that serum regulation of type I collagen expression is dependent upon adhesion interactions. It is possible that, rather than growth factors, adhesion components in serum such as fibronectin, vitronectin, and fibrinogen affect collagen expression in adherent cells by altering specific cell-ECM interactions. Thus, the lack of a serum effect in suspension may be due to the absence of these interactions.

Our preliminary data however, do suggest an exception to the anchorage dependence of collagen expression. Addition of transforming growth factor-β to cells maintained in suspension can reactivate collagen synthesis. It will be interesting to determine if transforming growth factor-β and adhesion activate collagen gene expression via similar regulatory systems.

Our studies suggest that the most apparent regulator of collagen gene expression in mouse fibroblasts is the extent of adhesion to a solid substratum. However, other parameters of cell morphology change as a result of the cell culture manipulations employed in these investigations. Most notable is the difference in overall cell shape. Studies in other systems have demonstrated a direct correlation between the extent of cell spreading and the synthesis of proteins that are characteristic of a particular cell phenotype. Generally, in these systems, a shift away from the flattened morphology leads to a more highly differentiated phenotype. Benya and Schaffer (1982) demonstrated that serial monolayer culture of rabbit articular chondrocytes results in a decrease in expression of cartilage specific type II collagen and an increase in interstitial type I collagen. This dedifferentiation event is reversed when cells are maintained in suspension culture in soft agar. Modulation of the shape of rabbit synovial fibroblasts by culture on surfaces of varying adhesivity also results in a change in type I collagen gene expression as well as the synthesis of metalloproteases, including collagenase and stromelysin (Aggeler et al., 1984). Recent studies in these shape-responsive systems reveal that regulation of gene expression responds more closely to the organization of the actin-containing cytoskeleton than the shape of the per se (Unemori and Werb, 1986; Benya et al., 1988).

The mechanism by which the cytoskeleton regulates specific programs of gene expression in these different cell types is unclear. It has been postulated that the intracellular matrix is involved in the transmission of second messenger signals to the nucleus. Many of the effectors that regulate the phenotype of a cell interact with cell surface receptors. Therefore, if the cytoskeleton is involved in signal transduction leading to the control of gene expression there must be communication between these receptors and the underlying intracellular matrix. There is a direct association of the cytoskeleton with the extracellular matrix through the integrin family of transmembrane receptors.

Binding of ECM molecules to integrins may directly activate second messenger systems and initiate a signal that is then transduced to the nucleus to influence gene expression. This notion was first validated by the observations that differentiation of myoblasts in culture can be prevented by the addition of a monoclonal antibody that inhibits binding of fibronectin to integrin (Menko and Boettiger, 1987). A recent study has now demonstrated that signal transduction through the fibronectin receptor regulates the expression of collagenase and stromelysin in rabbit synovial fibroblasts (Werb et al., 1989). These investigators revealed that either binding of fibronectin-derived fragments or adhesion blocking antibodies to the fibronectin receptor could induce gene expression. Adhesion to intact fibronectin, however, did not initiate a response. It is important to mention that these protease genes are activated when fibroblasts are deprived of adhesion or the adhesion structures are disrupted by cytochalasin D. In earlier studies (Aggeler et al., 1984), these investigators note that collagen expression responds in a reciprocal manner to the proteases, i.e. a decrease in expression in response to reduced adhesion.

The identification of adhesion-responsive genes is the first step in an analysis of the mechanisms that govern anchorage dependence. The activation of collagen expression by adhesion in serum-free conditions will permit us to analyze the role of specific ECM-receptor interactions with both soluble and insoluble ligands as well as to identify possible adhesion-responsive nuclear regulatory elements. In addition, we have found that several proteins are induced by suspension and down-regulated upon reattachment. Thus, this system allows an analysis of both positive and negative regulation by cell-ECM interactions.

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