Extracellular Matrix-Induced Gene Expression in Human Breast Cancer Cells

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Abstract
Extracellular matrix (ECM) molecules modify gene expression through attachment-dependent (focal adhesion-related) integrin receptor signaling. It was previously unknown whether the same molecules acting as soluble peptides could generate signal cascades without the associated mechanical anchoring, a condition that may be encountered during matrix remodeling and degradation and relevant to invasion and metastatic processes. In the current study, the role of ECM ligand-regulated gene expression through this attachment-independent process was examined. It was observed that fibronectin, laminin, and collagen type I and II induce Smad2 activation in MCF-10A and MCF-7 cells. This activation is not caused by transforming growth factor (TGF)-β ligand contamination or autocrine TGF involvement and is 3- to 5-fold less robust than the TGF-β1 ligand. The resulting nuclear translocation of Smad4 in response to ECM ligand indicates downstream transcriptional responses occurring. Coimmunoprecipitation experiments determined that collagen type II and laminin act through interaction with integrin α2β1 receptor complex. The ECM ligand-induced Smad activation (termed signaling crosstalk) resulted in cell type and ligand-specific transcriptional changes, which are distinct from the TGF-β3 ligand-induced responses. These findings show that cell-matrix communication is more complex than previously thought. Soluble ECM peptides drive transcriptional regulation through corresponding adhesion and non-attachment-related processes. The resultant gene expression patterns correlate with pathway activity and not by the extent of Smad activation. These results extend the complexity and the existing paradigms of ECM-cell communication to ECM ligand regulation without the necessity of mechanical coupling. (Mol Cancer Res 2009;7(3):319–29)

Introduction
In breast cancer development, extracellular matrix (ECM) regulates gene expression and phenotype through adhesion-mediated signaling (1, 2). A strong body of evidence indicates the importance of this process in many aspects of tissue homeostasis regulation from stromal fibroblast activation (3) to epithelial-to-mesenchymal transformation in tumorigenesis (4). Previous studies have focused on the role of ECM as a signal initiator in the context of an adhesion-related process. Tissue remodeling and protease degradation generates neopeptidases from ECM components that potentially act as “soluble” peptides in the pericellular microenvironment (5-8). These neopeptidases have been reported to induce changes in migration and cell behavior in some experimental systems (9-16). Although matrix effects are recognized, the contribution these peptides make to cellular phenotype in breast cancer is unknown. This potentially complements cytokines that are liberated and activated during tissue remodeling such as transforming growth factor-β (TGF-β), which is involved in epithelial-to-mesenchymal transformation (17).

TGF-β isoforms are produced and deposited into the ECM as inactive complexes by many cell types (18). Ligand activation can be achieved by several mechanisms, including through integrin αvβ6 and αvβ3 receptor interactions that liberate them for receptor binding (19, 20). Signaling occurs when TGF-β isoforms bind and activate the TGF-β receptor complex (type I and II), which is subsequently endocytosed and phosphorylated Smad2 and Smad3 proteins at their COOH-terminal SSXS amino acid sequence (21-25). Only ALK4, ALK5, and ALK7 receptors propagate signaling through Smad2 and Smad3 recognizing inputs from activin A, GDF1, GDF11, Nodal, and TGF-β ligands (26-28). These kinases are sensitive to SB-431542 inhibition with IC50 values 140 and 94 nmol/L (ALK4 and ALK5) and ~1 μmol/L (ALK7), respectively (29). All previously reported Smad-dependent TGF-β signaling events require TGF-β ligands for receptor activation. Novel collagen type II (CII) and angiotensin II-related Smad activation mechanisms have been reported (30, 31). It remains unknown whether the collagen-mediated process (α) is active in epithelial cells, (b) exists for other ECM molecules, (c) is independent of TGF-β ligand, and (d) has specific transcriptional consequences. The current article...
reports the consequences of soluble ECM-induced Smad2 activation. It characterizes CII and laminin (LAM) effects on the TGF-β/BMP signaling and pathway-specific transcriptional responses in MCF-10A “normal” and MCF-7 (estrogen receptor-positive) invasive human breast cancer cell lines. The results indicate that (a) soluble fibronectin, LAM, collagen type I (CI), and type II (CII) induce Smad2 phosphorylation, which is limited in magnitude if compared with native signaling; (b) this activation induces Smad4 nuclear translocation; (c) resultant Smad activation modulates gene expression in a ligand- and cell type-specific manner, which is distinct from TGF-β1 induced responses; and (d) this activity cannot be attributed to TGF-β contamination of ECM preparations.

Results

Smad2 Is Activated by ECM Treatments

LAM is a major component of basal membrane surrounding the acinus and breast epithelial cells. To mimic the effect of matrix degradation and cellular remodeling, we investigated how protease-digested LAM, fibronectin, CI, and CII peptides can effect Smad2 phosphorylation (Fig. 1A and B). Whereas fibronectin does not induce Smad2 phosphorylation in MCF-7 cells, MCF-10A cells display a 38% increase in Smad2 activation when compared with untreated cells (Fig. 1A). Both cell lines respond to CI with Smad activation, which is 33% greater in MCF-7 than in MCF-10A. The CI, CII, and LAM peptide-induced responses are 76%, 50%, and 72% in MCF-7 and 15%, 34%, and 24.5% in MCF-10A, respectively, from the unstimulated controls. In comparison, TGF-β1 results in a 400% Smad2 activation in both cell types. We chose to investigate CII and LAM effects further because α5β1 integrin receptors are the major binding complex for both peptides. Smad activation kinetics was compared at periods between 0 and 120 minutes following CII and LAM exposure (Fig. 1B). MCF-10A responses to soluble ECM peptides are greater in magnitude (LAM = 347% and CII = 350%) than MCF-7 (LAM = 207% and CII = 29.3%) densitometry at 120 min; right). pSmad2 levels gradually increased as a result of exposure, with the exception of MCF-7 CII, which peaks earlier at 45 min. All ECM Smad2 activations are significantly lower in magnitude than in TGF-β1-initiated responses. Similar responses were documented with JJ012 and 105KC chondro-sarcoma, C28 chondrocyte, Mv1Lu mink lung epithelial, and WM35 melanoma cell lines with Smad2 and Smad3 activation kinetics (data not shown). To confirm further that Smad2 activation is dependent on ALK4, ALK5, and/or ALK7 kinase sensitivity to TGF-β1 receptor inhibitor SB-431542 was determined (Fig. 1C). As shown, the inhibitor completely abolishes the ECM-induced Smad2 phosphorylation while significantly down-regulates the native TGF-β1 ligand-induced responses, indicating that both pathways require this kinase activity.

Crosstalk Signaling Is Independent from TGF-β Peptides, Mobilizes Full Pathway Activation, and Involves Integrin α5β1 Complex

The Smad2 activation by ECM samples raised the question whether the result was caused either by TGF-β contamination or the contribution of endogenously produced cellular inactive TGF-β. To address this concern, the Smad activation dynamics was reexamined with CII treatment in the presence of pan-specific TGF-β-neutralizing antibody (Fig. 2A; ND30 against hTGF-β1, pTGF-β1,2, pTGF-β2, rTGF-β3, and rTGF-β5 as 5.0, 1.0, 15.0, 4.0, and 1.0 μg/mL, respectively). Only TGF-β1 induction was down-regulated by the antibody (compare pSmad2 bands in AB-100-NA/TGF-β1 with TGF-β1 exposure). Contrary to this, ECM treatment-induced activation was not affected (AB-100-NA/CII versus CII), indicating that endogenously produced TGF-β isoforms did not contribute to the ECM-induced Smad activation. The possible TGF-β contamination of ECM peptides was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. No TGF-β1 contamination was detectable in the ECM peptide samples (4.0-14.0 μmol/L ECM sample loaded; 0.4 μmol/L TGF-β1 was the reference control, with instrumental sensitivity in femtomolar/attomolar range; data not shown).

The ECM peptide-induced Smad activation capacity prompted the analysis of Smad4 nuclear translocation (Fig. 2B) to verify that the peptide-induced Smad activation is capable of initiating downstream events. As shown, in untreated cells, Smad4 is not present in the nuclei, whereas ECM ligand and TGF-β1 exposure cause nuclear translocation of the signal.

The binding of CII and LAM to the integrin α5β1 receptor complex was confirmed by coimmunoprecipitation experiments in MCF cells (Fig. 2C). Integrin β1 antibody precipitates integrin α2 receptor independently from ligand exposure (bottom lane, related densitometry right). Its presence increased with combined peptides by 53.84% (far right, CII/LAM response IP bands 2), indicating that additional α5β1 receptor populations were accessible for complex formation and binding. This result is complemented by ligand competition, in which the detected CII and LAM decreased by 27.1% and 27.79%, respectively (top lane, CII/LAM; middle lane, band 2). These results indicate that CII and LAM are in competition for integrin α5β1 receptor binding. The lysis (bands = 1) shows the controls of appropriate targets in the total cell lysate. Immunoprecipitation experiments in MCF-10A cells duplicated the results observed in MCF-7 cells (data not shown).

Comparison of Gene Regulation Differences in MCF-10A and MCF-7 Cells

Because Smad2 activation was documented with the phosphospecific antibody recognizing only the double-phosphorylated (S465/S467) molecule and this induced Smad4 nuclear translocation, there was an expectation that the pathway activity will cause Smad-related gene expression changes, which can be analyzed by the TGF-β/BMP pathway-specific expression quantitative PCR array. It is also equally important how these pathway-specific genes are regulated differentially between MCF-10A normal and MCF-7 (estrogen receptor-positive) invasive untreated human breast cancer cells. Specific TGF-β/BMP signaling pathways were used to characterize cellular responsiveness to each of the ECM peptides. A 4 h time point was chosen to include still the stable early gene activation events together with the lasting mid-time and late regulations but
exclude the transient fluctuations. The cellular expression of 84 genes in MCF-7 was compared with MCF-10A cells (Fig. 3). The MCF-7 expression profile changes show fundamental up-regulation in five genes (BMP7, >CDKN2B, >PDGFB, >GSC and >BAMBI) and major down-regulation in six genes (INHA, >TGF-BR3, >TGF-BI, >PLAU, >NOGGIN, and >ENG; details are provided in Supplementary Figure and Table S1). The comparison shows that 63% (53 of 84) of genes are regulated.

**FIGURE 1.** MCF cells were handled, plated, synchronized, treated, and harvested as described in Materials and Methods. A. Smad activation (pSmad2) was tested without ligand (at 0 ng/mL) and with fibronectin (FN), CI, CII, and LAM (all at 50 μg/mL) and TGF-β1 (at 10 ng/mL) at 45 min. B. Time curve for Smad activation is comparable with the measured activation kinetics with the observation of limited effectiveness in accumulation of generated pSmad2 signal by ECM treatments. Compare MCF-7 CII and LAM treatments (pSmad2 and Smad2 lanes) with MCF-10A CII and LAM and their representative densitometry results (right). Significance of ECM treatments was analyzed on raw images acquired by UVP Bio-Imager (supplied by the software) in triplicates and then subjected to one-way ANOVA analysis (MATLAB 7.5.0) to establish the probability values (P). P < 0.001, difference in densitometry results of A and B. C. p100 plates were preincubated with SB-431542 for 30 min at 5.0 μmol/L final concentration at 37°C in the presence of 5% CO2 before pathway induction with CII and TGF-β1 exposures as above. Parallel samples were then harvested at indicated time points, pelleted, snap-frozen in liquid nitrogen, and stored at −80°C until use. Standardized total protein (125.0 μg) was subjected to SDS-PAGE analysis and Western blotting.
differentially in MCF-7. From these, 13 (25%) genes are upregulated and 40 (75.5%) genes are down-regulated. Particularly, the adhesion and extracellular molecules group are affected strongly, where 83% of genes are down-regulated from the affected 18 genes, suggesting that MCF-7 is less dependent on adhesion-related functions than MCF-10A.

**ECM Treatments Generate Distinct Expressional Responses**

The heat maps display absolute transcript levels of untreated controls (CTRL), CII, LAM, and TGF-β1 treated plates (Fig. 4). The results show that CII, LAM, and TGF-β1 regulate different sets of genes depending on ligand exposure and cell types. The

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**FIGURE 2.** Cells were treated as described in Materials and Methods. A. Parallel plates were pretreated with pan-specific AB-100-NA TGF-β1 neutralizing antibody (R&D Systems; ND50 against hTGF-β1, pTGF-β1, pTGF-β2, pTGF-β3, and pTGF-β5 as 5.0, 1.0, 15.0, 4.0, and 1.0 μg/mL, respectively) and used 25 μg/mL concentrations for 2 h (room temperature) to neutralize exogenously added and endogenous cellular production; the standard activation time curve was established with 50 μg/mL CII and 2.5 ng/mL TGF-β1 as control. For Smad4 nuclear translocation, MCF-10A and MCF-7 cells were plated at 3 × 10^5 per well concentration in six-well plates and synchronized overnight in serum-free medium and then treated as above.

B. Following 1 h incubation, the cells were washed, fixed, and processed with Smad4 primary antibody overnight followed with Alexa 488 secondary antibody for 2 h. Images were acquired on Zeiss AxioII microscope with GFP/FITC filter set.

C. CII and LAM are the major peptides for integrin α2β1 receptor complex. After lysis and quantitation, 2.0 mg total protein was subjected to integrin α2β1 pull-down for 2 h and then precipitated overnight at 4°C. ECM treatments are competing (signal down-regulation is <50%) for the same receptor population by combined CII/LAM treatments ([detecting antibodies (left) and pretreatments (right)] with CII detection (top) and LAM detection (middle); left and middle, corresponding densitometry). For validation of the right receptor complex pull-down, integrin α2 detection was used ([integrin β2 (bottom)]; IP bands 2, treatments, and CII, LAM, CII/LAM, and integrin β2 densitometry (right)). Respective Smad2 bands are generated by stripping and reprobing the membranes. Western blots were repeated in duplicates and corresponding densitometry ([right] analysis of raw acquired images (UVP imager software) was normalized to Smad2 signals. Statistical analysis was done as in Fig. 1. P = 0.0001, highly significant.
LAM induces more dynamic alternation in gene expression than CII or even TGF-β1 (CTRL versus LAM, CII, and TGF-β columns in MCF-10A/MCF-7 cells). Specifically, the BMP7, CDC25A, and COL3A1 genes show fundamental up-regulation in MCF-7 and remain responsive to LAM treatment only. The 84 genes are functionally grouped into five major areas (right) according to the assay description (PAHS-035; SA Bioscience). The comparison indicates that overall responsiveness to TGF-β signaling is down-regulated in MCF-7 cells, TGF-β isoforms 1, 2, and 3, ACVR1 and ACVR2A receptors, and Smad3 and Smad4 transmitters are all down-regulated, and the pathway inhibitor BAMBI is up-regulated.

Whereas the TGF-β/BMP signaling-specific pathway array focuses on expressional changes related only to Smad signaling activity and CII, LAM engagement with their integrin receptors are also inducing parallel signaling pathways. How the ECM peptides affected overall signaling activity in MCF-10A and MCF-7 cells was determined with a signal transduction pathway finder array (PAHS-014; Fig. 5). Overall, in normal MCF-10A cells, CII, LAM, and TGF-β1 response patterns are minimally overlapping (comparison of MCF-10A columns), indicating that the treatments differentially affect specific signaling pathways in these cells. Interestingly, the invasive MCF-7 cell line responds to CII and TGF-β1 in similar but not identical ways (note that there is no contamination or endogenous TGF-β1 ligand involvement in the ECM-induced Smad activation). The LAM affects different pathways and to a different extent (comparison of MCF-7 columns). All three treatments activated the CREB pathway similarly.

Validation of Crosstalk Sensitive Genes

To show that individual gene expression change depends on crosstalk and/or TGF-β1 ligand induction and pathway activity, selected genes from the TGF/BMP signaling and signal transduction pathway finder arrays were chosen for further expressional analysis in the presence of TGF-β1 receptor inhibitor (A-083-01; Fig. 6). ECM-induced and inhibitor-sensitive genes (Fig. 6A) represent the crosstalk-modulated segment of expressional patterns. The classic examples of TGF-β1 regulation are shown on FST (+19.810), MMP10 (+25.503), and SERPINE1 (+75.790; Fig. 6B). They are not sensitive to LAM and up-regulated by TGF-β1 and the inhibitor TGF-β1 + A-083-01 reverses the effect. The LAM + A-083-01 combination shows down-regulation similar to TGF-β1 + A-083-01, showing that LAM indeed does not affect these genes. Selected genes responding to LAM induction (Fig. 6C) are CCND1 (+/C0 7.17), FASN (-6.51), FN1 (+8.895), IGBP3 (+5.848), MYC (+/C0 15.63), and NOG (+/C0 28.797), which were then reversed by the inhibitor pretreatment (LAM + A-083-01), whereas neither TGF-β1 nor its combination with A-083-01 do not affect them. The exception is NOG, where LAM-induced down-regulation was reversed by the inhibitor (LAM + A-083-01) but was still negative (-8.717), whereas TGF-β1 does not affect NOG (1.149) and the inhibitor combination (TGF-β1 + A-083-01) down-regulates it (-18.65).

Supplementary Material

The functional comparison of untreated MCF-10A normal and MCF-7 cells was generated by Venn diagram (4 groups).
FIGURE 4. Real-time PCR with SYBR Green Master Mix was used to quantify the expression levels of 84 genes ontologically related and regulated by TGF-β/BMP signaling pathway or the 84 genes of the signal transduction pathway finder arrays (SA Biosciences). The heat map shows absolute mRNA copy numbers, which were calculated from PCR cycle thresholds (Ct). For example, on the color-coded log2 scale, a value of 10 represents \(2^{10} \) or 1,024 transcripts. Two endogenous controls, GAPDH and ACTB, were used for normalization. Right, functional gene clustering (with major groups according to the array manual).
presenting fold up- and down-regulation of genes in MCF-7 relative to MCF-10A normal control (Supplementary Fig. S1). The Venn groups, the fold differences, and related Student’s T-test, \( P \) values are listed in the Supplementary Table S1. The results presented in Figs. 3 - 6 were validated with independent TaqMan quantitative PCR assays designed against selected targets (Supplementary Table S2). Overall 31 genes were validated in independent assays. The differences between the TaqMan and SuperArray assays (SA) are greater when the transcript levels change dramatically (for example in BMP7 and CDKN2B), because the TaqMan assays are more target sensitive than the SYBR Green detection. Some of the genes are present in both arrays as CDKN2B, CDKN2A, JUN, IL2, FOS, and others. The validation proves that the array gene expression data are legitimate and accurate.

**FIGURE 5.** In the signal transduction pathway finder array, fold expression differences were analyzed through the SA Biosciences Web page, transferred into MATLAB, and visualized with the Bioinformatics Toolbox Clustergram function. All experiments were run in triplicates.
Discussion

The progression of breast cancer is associated with an epithelial-to-mesenchymal transition and involves components of TGF-β signaling (32, 33) and signaling from the ECM (34-37). MCF-10A and MCF-7 (estrogen receptor-positive) cell lines are well-established model systems for the study of tumor microenvironment in breast cancer progression (38). In addition to the influence of the ECM, the capacity of neoepitopes created by protease digestion of matrix molecules to modulate cell migration has also been reported (15). The signaling induced by proteolytic fragments of ECM proteins is largely unknown. Using this model system, we show that soluble ECM peptides induce Smad2 activation in human breast cancer cells. The role of integrin-mediated signaling is well recognized (39) and in conjunction with this study suggests that a signaling outcome depends on the modulation of a signaling network rather than affecting a single pathway. The perceived complexity of cell-ECM communication and matrix-originated signal transduction was previously based on the assumption that these processes are all related to adhesion with an immobile matrix (40). To extend these original paradigms, this study shows that soluble ECM molecules activate Smad signaling by binding to their representative integrin receptors and, in parallel, indirectly activating the TGF-β signaling pathway.

The pSmad2-specific antibody that was used recognizes the dual phosphorylation of COOH-terminal SSXS motif. This activation of Smad2 and Smad3 is described only by the ALK4, ALK5, and ALK7 receptors (22, 27, 41). The demonstrated TGF-β1 receptor inhibitor (SB-431542) sensitivity of both pathways verifies ALK5 involvement without excluding the possible participation of ALK4 and ALK7. The detailed molecular mechanisms by which the ECM and native TGF-β ligand-induced Smad activation overlaps and differs are beyond the scope and focus of this current manuscript. The fact that this ECM-induced signaling can be observed within 15 min after ligand exposure is in good agreement with the known kinetics for Smad activation by TGF-β ligand (21, 42).

FIGURE 6. Parallel triplicate experiments were plated and synchronized as described in Materials and Methods. A-083-01 (5 μmol/L) pretreatment was used (30 min) on selected samples followed by LAM and TGF-β1 exposure alone and in combination with the inhibitor. Samples were harvested after 4 h incubation to enhance the stable expressional profile changes. Selected genes were assayed on cDNA library generated (Materials and Methods) by ABI TaqMan probes (Supplementary Table S2) on ABI 7900 HT Fast Real-time Quantitative PCR instrument. Results were transferred to Excel (Microsoft). Ct values from three independent experiments. Bars, SD. A, Crosstalk process (green). B, TGF-β responsive genes. C, Crosstalk responsive genes.
The integrin α2β1 complex is a major receptor for both LAM and CII and malignant ligand (43). It is also implicated in mediating the malignant transformation in pancreatic cancer cell lines (44). Moreover, the LAM is particularly important in MCF cells for acinus development as a scaffolding matrix (Matrigel) as well as medium component (45). The coimmunoprecipitation experiments confirmed that CII and LAM are competing ligands for integrin α2β1 and binding increases the association of the heteromeric receptor complex. The soluble ECM-induced Smad activation (termed as crosstalk signaling) was verified in multiple cell lines, indicating that this process can represents a general mechanism for ECM molecules when acting as soluble peptides.

The TGF-β signaling is tightly controlled by the ECM through regulating the availability of free peptides for TGF-β receptor activation (46). It can be hypothesized that cell binding to ECM could synergistically enhance the binding of latent TGF-β binding protein and latency-associated protein complexes to their integrin αvβ6 and α2β1 receptors and release the peptides by the ECM stimuli to initiate Smad signaling. This scenario can be excluded for three reasons. (a) The observed ECM-Smad activation kinetics makes it unlikely and would require an interaction between α2β1, αvβ6, TGF-β1, and TGF-β2 receptors plus latency-associated protein and latent TGF-β binding protein complexes, and the ECM-induced Smad signals can be detected within 15 min. (b) We show that the AB-100-NA neutralizing antibody blocks only the TGF-β1-induced signaling but not affecting the crosstalk itself, indicating that the two processes are separate. (c) No TGF-β isoform contamination was found by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Moreover, if Smad activation by ECM is a result of TGF-β1 ligand participation, it would be expected that ECM exposure activates the same genes with perhaps lower amplitude depending on the signaling thresholds as TGF-β1 does.

Contrary to this, each ECM ligand elicits a different set of expression patterns when compared with each other and with TGF-β1. MCF cells are regulated by collagen type IV and LAM but normally do not encounter CII in their in vivo environment (38). Therefore, the CII is an ideal control to show that just because it binds to the same integrin α2β1 complex, activating the same crosstalk and integrin signaling pathways, the expression responses are still separated and selective for the representative treatments. For example, the CII-induced Smad signaling in the MCF-7 cells peaks at 45 min (Figs. 1 and 2), and with greater magnitude than the corresponding LAM signal. However, this is not capable of generating any significant expression differences (Fig. 4, MCF-7 panel CTRL versus CII), whereas, in MCF-10A, at the same level and time, the CII-related Smad signal regulates ~11% of genes from the total of 84 genes (MCF-10A panel CTRL versus CII). This suggests that gene expression responses are ligand and cell type dependent and therefore unlikely to be the result of TGF-β1 ligand; both LAM and CII induce similar pSmad2 levels but elicit different separate transcriptional responses.

The data indicate that the MCF-7 cells are also less responsive to TGF-β signaling than MCF-10A. The comparison of untreated MCF-10A and MCF-7 cell lines shows that 63% (53 of 84) of genes are differentially regulated in MCF-7. From these, 13 (25%) genes are up-regulated and 40 (75.5%) genes are down-regulated. Notably, the adhesion and extracellular molecule clusters are affected strongly, where 83% of genes are down-regulated from the affected 18 genes, indicating that MCF-7 is less dependent on adhesion-related functions than MCF-10A (Supplementary Figure). The regulation of this selective target gene population reflects the increase of invasive capacity of MCF-7 cells when compared with MCF-10A line.

The LAM-induced expression changes were validated on selected genes displaying crosstalk sensitivity (Fig. 6; genes respond to LAM induction, which then reversed by A-083-01 TGF-β1 receptor inhibitor) by ABI TaqMan probes. As shown, the inhibitor selectively blocks TGF-β1-induced FST, MMP10, and SERPINE1. These are the classic responsive genes of TGF-β pathway activity. Furthermore, the inhibitor also reverses genes regulated by LAM (not TGF-β1), verifying that the fold expression change of these genes was indeed induced by LAM (crosstalk) throughout the TGF-β pathway and not by alternative branch of attachment-dependent integrin signaling.

This article shows that ECM molecules induce transcriptional responses through a non-attachment-related signaling process that parallels classic integrin signaling. This process affects cellular pathways on a ligand- and cell type-dependent manner in human breast cancer and other cells that were investigated. The invasive MCF-7 cells show similar but not identical responses to CII as to TGF-β1, whereas the LAM response patterns are distinct from both. Crosstalk process modulates the signaling capacity of the invasive MCF-7 cells, which has been shown be fundamental in epithelial-to-mesenchymal transition and breast cancer progression (17, 47, 48).

Consequently, the ECM-cell communication affects transcriptional regulation in a way that is more complex than previously thought. Furthermore, when matrix components act on attachment-independent way (during matrix remodeling and degradation), this process can affect gene expression and contribute to ECM-originated signaling, controlling cellular activity and phenotype.

Materials and Methods

Cell Culture

MCF-10A cells (human normal mammary epithelial cells) were propagated in MEGM (Clonetics; with supplied Single-Quots growth factors) with 100 ng/mL cholera toxin (Calbiochem). MCF-7 cells were cultured in IMM (Life Technologies; plus gentamicin) containing 10% fetal bovine serum, 10 mg/L phenol red (Sigma), and 10 μg/mL insulin (Sigma).

Chemicals

Fibronectin (Sigma), CI (Sigma), and CII (Chondrex) was dissolved in 0.05 mol/L CH3COOH as 1.0, 2.0, and 2.0 mg/mL stock solutions, respectively. The LAM (Sigma) was supplied as 1.0 mg/mL stock in 50 mmol/L Tris-HCl (pH 7.5) with 150 mmol/L NaCl, TGF-β1 (R&D Systems) stock was 10 ng/μL in 4.0 mmol/L HCl with 1.0 mg/mL bovine serum albumin carrier protein. SB-431542 and A-083-01 were purchased from Tocris Bioscience.
Antibodies

The primary antibodies were purchased from Cell Signaling (anti-p-Smad2 and anti-Smad2) and Santa Cruz Biotechnology (anti-Smad4, CII, LAM, and integrin β1 and α5). Secondary horseradish peroxidase-conjugated antibodies are from Santa Cruz Biotechnology and Amersham/GE. Anti-mouse Alexa 488 is from Molecular Probes/Invitrogen.

ECM Treatments

Cells were plated in p100 plates at density of $6.0 \times 10^5$ (MCF-10A) to $8.0 \times 10^5$ (MCF-7)/p100 to give a confluent culture after overnight incubation. The cultures were synchronized by serum-free DMEM:F-12 for 24 h to maximize the signal-to-noise ratio. ECM peptides were applied at $50 \mu$g/mL concentration in 4 mL serum-free DMEM:F-12 for the specified time. TGF-β1-positive control was used at $10 \mu$g/mL concentration. To harvest, cells were washed twice on ice with ice-cold PBS, scraped in 1 mL PBS, and pelleted at 8,000 rpm for 2 min, and the pellet was snap-frozen in liquid nitrogen and stored at $-80^\circ$C until processing.

Western Blotting and Image Acquisition

Cells were lysed in buffer containing 50 mmol/L Tris-Cl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonyl fluoride completed with protease inhibitor cocktail from Roche. The samples were normalized for protein with ND-1000 (NanoDrop Technologies). For Western blotting, 125 μg protein/lane were analyzed with primary antibodies incubated overnight at 4°C followed by secondary horseradish peroxidase-conjugated antibodies for 2 h at room temperature. Bands were detected with SuperSignal West Pico ECL detection kit (Pierce) on UVP Biospectrum Digital Imaging system (UVP). The raw images were quantitated by absorbance through the supplied densitometry analysis software and normalized to total Smad levels.

Coimmunoprecipitation

Confluent synchronized p100 plates were pretreated for 2 h at 4°C with CII and LAM and in combinations. Then, the cells were harvested and samples were stored as described above. Samples were processed in 800 μL lysis buffer for 30 min on ice. Supernatants were separated, and the residual pellets were processed again with a fresh aliquot (200 μL) and sonicated (converter V2391, Virsonic 100 unit VirTis; SP Industries). The combined supernatants were quantitated and 2.0 mg total protein samples were incubated with mouse anti-human VLA-2 (α5β1; Chemicon) against functional collagen receptor or mouse anti-human integrin β1 (Santa Cruz Biotechnology) for 2 h and then precipitated with 45 μL protein A agarose (Sigma), anti-mouse IgG (Sigma), or protein G agarose at 4°C overnight. Samples were washed extensively, solubilized in 80 μL of 2× Laemmli buffer, and analyzed on 8% SDS-PAGE.

Gene Expression Analysis

Human TGF-β/BMP signaling (PAHS-035) and signal transduction pathway finder RT² profiler PCR arrays were obtained from SA Bioscience. Cells were plated in triplicates, exposed to peptides, harvested at 4 h later, and stored as described above. RNA was purified with RNeasy Mini Kit on column DNase treatment (Qiagen) according to the manufacturer’s protocol. For the cDNA synthesis, 5 μg total RNA was used with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described by the kit manual. The array analysis was carried out following the manufacturer’s protocol with SYBR Green PCR (ROX) Master Mix (Applied Biosystems) and 1.0 to 5.0 μg cDNA/plate on ABI 7900HT Fast Quantitative PCR System. Gene expression differences were determined using the 2^−ΔΔCt method according to AB and SA Biosciences protocols.

The expression pattern differences between the MCF-10A and MCF-7 cells and the heat maps were generated in MATLAB 7.5.0 software using the expression fold differences analyzed through the SA Biosciences Web page, and with absolute values of the transcripts were measured by quantitative PCR.

Statistical Analysis

One-way ANOVA subroutine of MATLAB 7.5.0 was used to verify the significance of Western blotting results (quantitated through the UVP Bio-Imager densitometry software in triplicates). The array analysis-related P values were generated through the representative Web page links supported software. Values are presented in the Supplementary Material.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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