

## Integrins $\beta_1$ , $\alpha_6$ , and $\alpha_3$ contribute to mechanical strain-induced differentiation of fetal lung type II epithelial cells via distinct mechanisms

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**Sanchez-Esteban, Juan, Yulian Wang, Edward J. Filardo, Lewis P. Rubin, and Donald E. Ingber.** Integrins  $\beta_1$ ,  $\alpha_6$ , and  $\alpha_3$  contribute to mechanical strain-induced differentiation of fetal lung type II epithelial cells via distinct mechanisms. *Am J Physiol Lung Cell Mol Physiol* 290: L343–L350, 2006. First published September 16, 2005; doi:10.1152/ajplung.00189.2005.—Mechanical forces regulate lung maturation in the fetus by promoting type II epithelial differentiation. However, the cell surface receptors that transduce these mechanical cues into cellular responses remain largely unknown. When distal lung type II epithelial cells isolated from embryonic *day 19* rat fetuses were cultured on flexible plates coated with laminin, fibronectin, vitronectin, collagen, or elastin and exposed to a level of mechanical strain (5%) similar to that observed in utero, transmembrane signaling responses were induced under all conditions, as measured by ERK activation. However, mechanical stress maximally increased expression of the type II cell differentiation marker surfactant protein C when cells were cultured on laminin substrates. Strain-induced alveolar epithelial differentiation was inhibited by interfering with cell binding to laminin using soluble laminin peptides (IKVIV or YIGSR) or blocking antibodies against integrin  $\beta_1$ ,  $\alpha_3$ , or  $\alpha_6$ . Additional studies were carried out with substrates coated directly with different nonactivating anti-integrin antibodies. Blocking integrin  $\beta_1$  and  $\alpha_6$  binding sites inhibited both cell adhesion and differentiation, whereas inhibition of  $\alpha_3$  prevented differentiation without altering cell attachment. These data demonstrate that various integrins contribute to mechanical control of type II lung epithelial cell differentiation on laminin substrates. However, they may act via distinct mechanisms, including some that are independent of their cell anchoring role.

laminin; surfactant protein C

LUNG GROWTH AND DEVELOPMENT during fetal life are critical for extrauterine survival. Premature infants are often born before sufficient lung maturation has occurred with resultant increased morbidity and mortality. Lung maturation, in part, is controlled mechanically. For example, fetal thoracic movements, which generate ~5% changes of the distal lung surface area (14, 15), and cell tractional forces exerted on extracellular matrix (ECM) within tissues have been shown to be critical regulators of fetal lung development (28, 35, 43).

A key component of lung development is the differentiation of type II epithelial cells, the major source of pulmonary surfactant that prevents alveolar collapse during expiration. These cells also participate in fluid homeostasis in the alveolar lumen, host defense, and restoration of normal alveolar epithelium after lung injury (38).

Past studies have shown that application of levels of mechanical strain that simulate the cell distortion produced by fetal breathing movements induces fetal type II epithelial cell maturation and that the ERK pathway mediates this response (41, 43). However, the mechanisms by which lung cells sense mechanical forces via mechanoreceptors to activate this intracellular differentiation pathway remain largely unknown.

Interactions between pulmonary epithelial cells and ECM proteins such as laminin or fibronectin or different collagen subtypes modulate lung development (34). Laminin is a major glycoprotein component of epithelial basement membranes synthesized and secreted by lung epithelial cells (46). It is important for epithelial cell adhesion (32), branching morphogenesis (35, 45), and alveolar formation (32). Cell adhesion to laminin and other ECM components is mediated by transmembrane integrin receptors (10, 49, 52).

Integrins are a family of ubiquitous cell surface receptors that mechanically couple the ECM to the cytoskeleton (53) and control a variety of cell functions by serving as scaffolds for the assembly of multiprotein signaling complexes within focal adhesion anchoring sites (3, 8). Because integrins preferentially mediate mechanical force transfer across the cell surface (53), they are ideally positioned to sense mechanical stimuli and, through their interconnections with focal adhesion proteins, transduce them into biochemical signals to modify cell behavior (7, 18, 19, 33). Numerous studies have confirmed that integrins play a central role in mechanotransduction in virtually all cell and tissue types (1, 24, 40, 50).

The goal of the present study was to explore the role of integrins during pulmonary type II epithelial cell differentiation induced by mechanical stress. We used an *in vitro* model system in which fetal lung type II cells are cultured on flexible ECM substrates and exposed to a physiologically relevant level of mechanical strain similar to that experienced by type II cells in utero. These studies revealed that the ECM protein laminin preferentially mediates the effects of force on type II cell differentiation relative to other ECM molecules and that different integrin subtypes contribute to this response via distinct mechanisms.

### MATERIALS AND METHODS

**Experimental system.** Fetal rat lungs were obtained from timed-pregnant Sprague-Dawley rats (Charles River, Wilmington, MA), and

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embryonic *day 19* (E19) type II cells were isolated using a modified version of a published technique (43). After collagenase digestion, cell suspensions were sequentially filtered through 100-, 30-, and 20- $\mu\text{m}$  nylon meshes using screen cups (Sigma). The filtrate from the 20- $\mu\text{m}$  nylon mesh, containing mostly fibroblasts, was discarded. Clumped nonfiltered cells from the 30- and 20- $\mu\text{m}$  nylon meshes were collected after several washes with DMEM to facilitate the filtration of nonepithelial cells. Further type II cell purification was achieved by incubating the cells in 75- $\text{cm}^2$  flasks for 30 min. Purity of the type II cell fraction was determined to be  $90 \pm 5\%$  by microscopic analysis of epithelial cell morphology and immunostaining for cytokeratin and vimentin as markers of epithelial cells and fibroblasts, respectively (43).

Nonadherent cells were collected, plated on Bioflex six-well plates (Flexcell, Hillsborough, NC) precoated with different ECM proteins, and maintained for 24 h in serum-free DMEM. ECM proteins were applied to Bioflex plates by adsorption. Membranes were coated overnight with PBS solutions containing laminin-1 (2  $\mu\text{g}/\text{cm}^2$ ; Sigma, cat. no. L-2020), fibronectin (5  $\mu\text{g}/\text{cm}^2$ ; Sigma, cat. no. F-0635), vitronectin (0.5  $\mu\text{g}/\text{cm}^2$ ; Sigma, cat. no. V-0132), collagen-1 (10  $\mu\text{g}/\text{cm}^2$ ; Collagen Biomed, cat. no. PC0701), or elastin (10  $\mu\text{g}/\text{cm}^2$ ; Sigma, cat. no. E-6402). Plates were washed with PBS followed by BSA incubation (1 mg/ml) for 1 h at 37°C to block uncoated sites on the membranes. The plates were then rinsed with culture medium to remove unadsorbed proteins before experiments.

In experiments with immobilized antibodies, Bioflex plates were coated with blocking anti- $\beta_1$ -integrin antibody (10  $\mu\text{g}/\text{ml}$  in PBS, BD Transduction Laboratories, cat. no. 555002) or blocking anti- $\alpha_3$ -integrin antibody (Ralph3.1, clone 6B3, 10  $\mu\text{g}/\text{ml}$ ; Developmental Studies Hybridoma Bank, Univ. of Iowa) for 2 h at room temperature, rinsed with PBS, and incubated with 1% BSA in PBS for 1 h at 37°C. Before the substrates were coated with nonactivating anti- $\alpha_6$ -integrin antibodies (10  $\mu\text{g}/\text{ml}$  in PBS, Serotec, cat. no. MCA2034) for 2 h at room temperature, they were precoated with goat anti-mouse secondary antibody (50  $\mu\text{g}/\text{ml}$  in PBS, Jackson ImmunoResearch Laboratories). After rinsing the plates twice with PBS and once with DMEM, we seeded fresh isolated E19 cells on these antibody-coated substrates in the absence of serum and allowed them to adhere for 4 h before the application of mechanical strain.

Plates containing adherent cells were mounted in a Flexercell FX-4000 Strain Unit (Flexcell). Equibiaxial elongation of 5% was applied at intervals of 60 cycles/min for 15 min plus 2.5% continuous distention for the remaining 45 min of each hour for different lengths of time. This regimen was chosen to simulate mechanical forces experienced by type II epithelial cells during fetal lung development (36). Cells grown on nonstrained substrates were treated in an identical manner and served as controls.

In some studies, E19 cells were incubated with soluble laminin peptide or anti-integrin antibodies in suspension at 37°C for 15 min before being plated on laminin-1 substrates in the continued presence of these reagents in the following concentrations: IKVIV (50  $\mu\text{g}/\text{ml}$ ; Sigma, cat. no. C-6171), YIGSR (100  $\mu\text{g}/\text{ml}$ ; Sigma, cat. no. T-7154), RGD (100  $\mu\text{g}/\text{ml}$ ; Calbiochem, cat. no. 03-34-0029), blocking integrin  $\alpha_3$  (Ralph3.1, clone 6B3, 10  $\mu\text{g}/\text{ml}$ ; Developmental Studies Hybridoma Bank), and integrin  $\alpha_6$  (40  $\mu\text{g}/\text{ml}$ ; Serotec, cat. no. MCA2034).

**Assessment of ERK activation.** To measure ERK activity, cell monolayers were lysed with ice-cold RIPA buffer (150 mM NaCl, 100 mM Tris base, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 3.5 mM  $\text{Na}_3\text{VO}_4$ , 2 mM PMSF, 50 mM NaF, 100 mM sodium pyrophosphate) with protease inhibitors (10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  aprotinin, 143.5  $\mu\text{M}$  aminoethyl benzenesulfonyl fluoride). Lysates were centrifuged, and total protein contents were determined by the bicinchoninic acid method. Protein samples were separated by one-dimensional SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated for 1 h at room temperature in blocking buffer (25 mM Tris-Cl, pH

8.0; 1.25 mM NaCl; 0.1% Tween 20 with 5% nonfat dry milk) and then incubated with anti-phospho-ERK1/2 antibody (Cell Signaling, Beverly, MA) for 1 h at room temperature. After being washed, secondary antibody (donkey anti-rabbit horseradish peroxidase diluted 1:2,000 in blocking buffer) was added for 1 h at room temperature. Immunoreactive phospho-ERK1/2 was detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). To control for protein loading, membranes were stripped and reprobed with antibody to total ERK1/2 (Cell Signaling); the intensity of the bands was analyzed by densitometry.

**Northern blot analysis.** Total cellular RNA was isolated using a single-step method as previously described (42). Briefly, RNA was denatured at 65°C for 5 min and fractionated by 1.4% agarose and 2.2 M formaldehyde gel electrophoresis. RNA was blotted, transferred to GeneScreen (NEN, Boston, MA) nylon membranes, and immobilized by UV cross-linking. Surfactant protein C (SP-C) probe was synthesized from linearized recombinant phagemid template using *in vitro* transcription (Promega, Madison, WI), T7 RNA polymerase, and [ $\alpha$ - $^{32}\text{P}$ ]UTP (Amersham). Blots were hybridized with the SP-C probe and washed in  $0.5\times$  SSC/1% SDS. The intensity of mRNA bands of interest in each lane was normalized to 18S rRNA fluorescence to control for differences in sample loading and RNA integrity. Blots were exposed to X-ray films with intensifying screens at  $-80^\circ\text{C}$ ; autoradiographs were measured by densitometry.

**Cell adhesion assays.** The effects of specific anti-integrin antibodies on cell adhesion were measured in microtiter plates precoated with laminin-1 (2  $\mu\text{g}/\text{cm}^2$ ). Suspended cells were incubated with cycloheximide (20  $\mu\text{g}/\text{ml}$ ), washed, and incubated with anti- $\alpha_3$  (Ralph3.1, clone 6B3; 10  $\mu\text{g}/\text{ml}$ ; Developmental Studies Hybridoma Bank),  $\alpha_6$  (40  $\mu\text{g}/\text{ml}$ ; Serotec, cat. no. MCA2034), or  $\beta_1$  antibodies (40  $\mu\text{g}/\text{ml}$ ; BD Transduction Laboratories, cat. no. 555002) for 15 min at 37°C and then plated on the laminin substrates in the presence of the antibodies and cycloheximide. After 4 h, nonadherent cells were washed from the substrate with PBS. Cells were fixed with 4% paraformaldehyde in PBS and stained with crystal violet (Sigma, 0.1%) in ddH<sub>2</sub>O for 25 min at room temperature. After several washes with tap water, stained cells were solubilized overnight with 0.5% Triton X-100 (diluted in ddH<sub>2</sub>O), and the optical density was measured at 590 nm. Cell numbers were derived from a standard curve.

**Immunofluorescence microscopy.** Cultured cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Silastic membranes were mounted on glass slides, and cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min at room temperature. The samples were then incubated in blocking buffer (1 $\times$  Tris-buffered saline, 0.1% Tween 20, and 2% normal goat serum, in PBS) for 1 h at room temperature and incubated with primary antibody to SP-C (FL-197; Santa Cruz, cat. no. SC-13979) at 1:30 dilution in blocking buffer at 4°C overnight. Cells were washed and incubated in Alexa Fluor 488 goat anti-rabbit secondary antibody at 1:100 dilution (Molecular Probes) in the same buffer solution for 1 h at room temperature. Monolayers were then washed, mounted, and analyzed by fluorescence microscopy.

**Statistical analysis.** Results are expressed as means  $\pm$  SE from at least three experiments, using different litters for each experiment. Control and stretched samples were compared by unpaired Student's *t*-test. For multiple comparisons, data were analyzed with ANOVA followed by post hoc tests, and Instat 3.0 (GraphPad Software, San Diego, CA) was used for statistical analysis;  $P < 0.05$  was considered statistically significant.

## RESULTS

**Mechanical stress induces ERK activation on various ECM substrates.** The ERK signaling pathway plays an important role in stretch-induced type II cell differentiation (43). Based on the importance of cell-ECM interactions for mechanotransduction in other cell types (13, 21, 56), we examined whether different

ECM proteins differ in their ability to mediate mechanical strain-induced ERK activation in freshly isolated E19 type II epithelial cells. The cells were cultured on flexible silicone membranes coated with laminin-1, fibronectin, vitronectin, collagen-1, or elastin, and then subjected to a cyclical mechanical strain regimen (5% strain for 15 min/h + 2.5% strain continuously for the remaining 45 min of each hour) for 6 h that mimicked the forces experienced by type II cells in utero (36). These studies revealed that all five ECM proteins were able to mediate mechanotransduction in type II epithelial cells to a similar degree, as measured by activation of ERK at 6 h of stress application (Fig. 1).

**Strain-induced differentiation of type II cells is matrix dependent.** To explore the functional significance of these effects on ERK activation for type II cell differentiation, Northern blot analysis of mRNA expression of the type II cell-specific surfactant protein SP-C was carried out under similar conditions. These studies revealed that cells cultured on different ECM substrates but exposed to the same mechanical stress exhibited significantly different differentiation responses. Cells on laminin-1 and collagen-1 substrates exhibited the greatest increase (~3.5-fold) in SP-C mRNA expression, whereas cells on vitronectin were less sensitive, and those on fibronectin or elastin substrates displayed only a modest (<2-fold) increase (Fig. 2A). Similar strain-dependent induction was observed when total SP-C protein levels were assessed by immunofluorescence microscopy (Fig. 2B). Moreover, we showed that strain-dependent increases in SP-C expression were matched by increases in the expression of another marker of type II cell

function, SP-B, in a past study (41). Thus these experiments indicate that ECM proteins differ in their ability to support strain-induced differentiation of type II pulmonary epithelial cells.

**Soluble laminin peptides block stretch-induced SP-C expression.** Based on the finding that laminin preferentially mediated strain-induced type II cell differentiation, we tested whether blocking laminin-binding sites with soluble peptides would interfere with the cytodifferentiation response induced by strain. Cells preincubated with the laminin  $\alpha$ -chain peptide IKVAV, the  $\beta$ -chain peptide YIGSR, or the fibronectin peptide RGD were cultured in the presence of these peptides on laminin substrates and subjected to mechanical strain for 16 h. Inclusion of IKVAV or YIGSR in the culture medium almost completely abrogated the differentiation response, whereas the control RGD peptide had no effect (Fig. 3A). In contrast, none of these peptides interfered with cell adhesion (Fig. 3B) or spreading (data not shown) on laminin substrates.

**Type II cell adhesion to laminin-1 is mediated by integrins  $\beta_1$  and  $\alpha_6$ .** To determine which integrins mediate adhesion to laminin substrates, fetal type II cells were preincubated with different anti-integrin receptor antibodies and were then plated in the presence of these same blocking antibodies on laminin substrates. Addition of blocking antibodies directed against integrins  $\beta_1$  or  $\alpha_6$  significantly inhibited attachment, and the combination of both almost completely prevented cell adhesion (Fig. 4). In contrast, blocking antibodies against  $\alpha_3$  had no effect. Thus  $\beta_1$ - and  $\alpha_6$ -integrin subunits appear to be the primary mediators of fetal type II cell attachment to laminin-1.

**Integrin-dependent control of strain-induced type II cell differentiation.** Next, we examined whether these different integrin receptors differed in their ability to support stress-induced differentiation of type II cells. Inhibition of binding of integrin  $\alpha_6$  or  $\alpha_3$  using specific blocking antibodies decreased SP-C mRNA expression in mechanically strained cells when compared with stretched samples incubated with a nonspecific IgG control (Fig. 5A).

To further explore the role of specific integrin subunits in this cellular mechanotransduction response, cells were cultured on flexible membranes coated directly with different anti-integrin antibodies instead of with ECM proteins to constrain the integrin receptor through which the cell initially experiences the mechanical stress. When cells grown on substrates coated with integrin  $\beta_1$ ,  $\alpha_6$ , or  $\alpha_3$  were mechanically strained, SP-C mRNA significantly increased compared with unstretched controls (Fig. 5B), and these effects were matched by increases in expression of SP-C protein, as measured using immunofluorescence microscopy (Fig. 5C). These data corroborate the functional blocking experiments above and confirm that integrins  $\alpha_6$ ,  $\beta_1$ , and  $\alpha_3$  selectively mediate mechanotransduction promoting fetal type II cell differentiation.

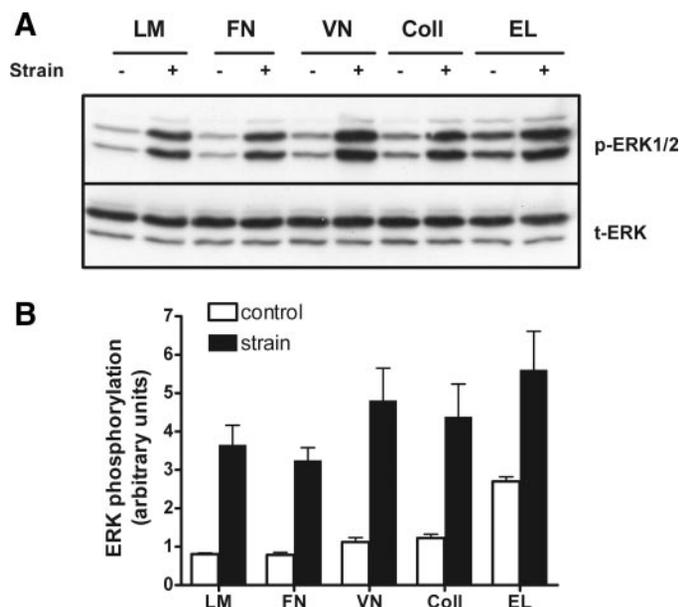


Fig. 1. A: Western blot showing that mechanical strain induces ERK activation in fetal type II pulmonary epithelial cells cultured on various ECM proteins. Type II cells isolated from embryonic day 19 rat fetal lung were cultured on flexible silastic membranes coated with laminin (LM), fibronectin (FN), vitronectin (VN), collagen-1 (Coll), and elastin (EL) and were then subjected to cyclical mechanical strain for 6 h. Proteins were extracted and the levels of ERK phosphorylation (p-ERK1/2) were analyzed using a phospho-specific ERK antibody; blots were then stripped and incubated with total ERK (t-ERK) antibody to control for protein loading. B: results from 3 experiments showing that mechanical strain phosphorylates ERK to a similar degree in cells cultured on each of the 5 ECM substrates.

## DISCUSSION

The best-characterized differentiated function of fetal alveolar type II cells is the synthesis and secretion of pulmonary surfactant. In the present study, we analyzed the effects of mechanical stress on fetal type II cell differentiation by measuring changes in the mRNA and protein levels for the surfactant protein SP-C. SP-C mRNA and precursor protein have been detected in embryonic lung epithelial cells (55, 57), and

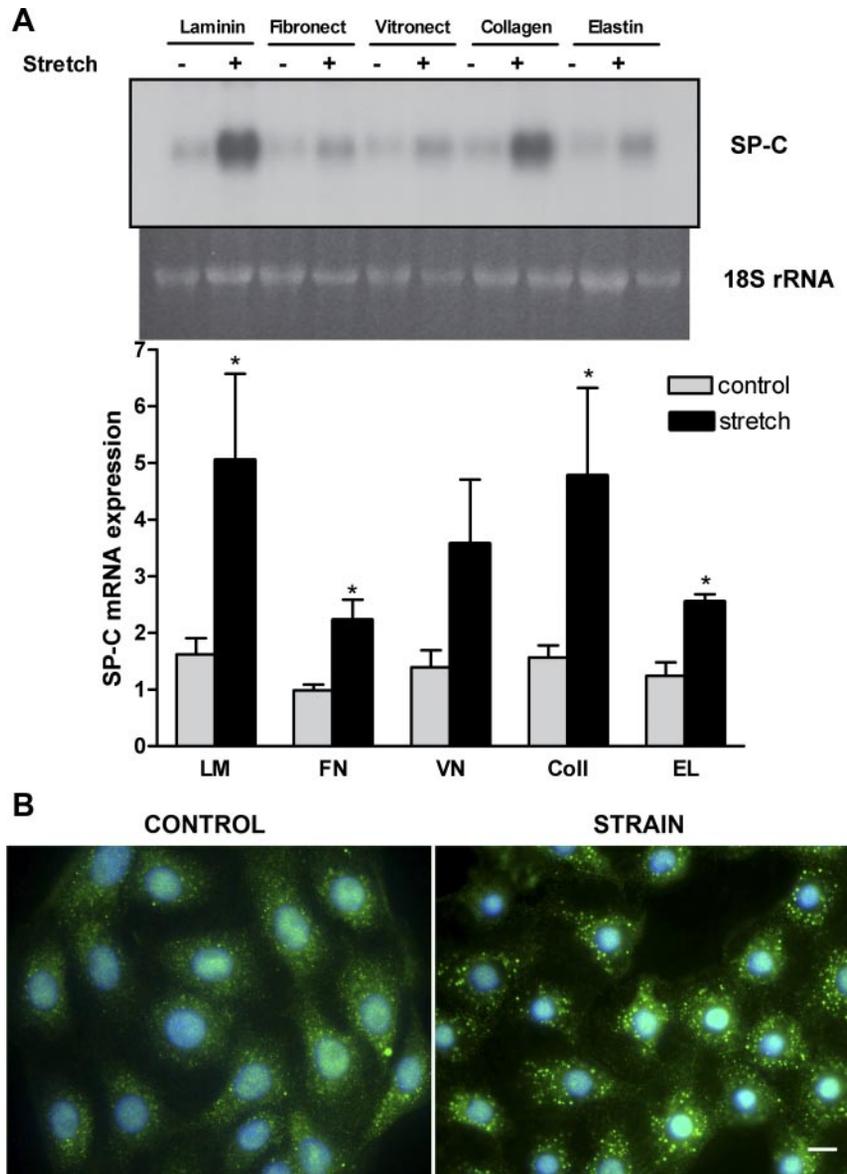


Fig. 2. *A, top*: Northern blot of surfactant protein C (SP-C) mRNA expression showing that strain-induced type II cell differentiation is matrix dependent. Shown is a representative blot obtained from cells cultured on different ECM proteins and exposed to same cyclical strain regimen assayed for SP-C and 18S ribosomal RNA accumulation after 16 h. *A, bottom*: results from 3 experiments demonstrating that the degree of SP-C mRNA expression induced by mechanical deformation differs depending on the ECM substrate used for cell attachment. \* $P < 0.05$ . *B*: fluorescence immunocytochemistry images demonstrating that mechanical strain of fetal type II cells on laminin-1 substrates increases SP-C protein levels (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Bar, 10  $\mu$ m.

their expression levels increase with advancing gestational age, so that by term, SP-C is only expressed in alveolar type II cells (25). The SP-C gene promoter also has been shown to be specific for type II cells (12). Our results demonstrate that different ECM molecules and integrin subunits differentially mediate the effects of mechanical strain on fetal type II lung epithelial cell differentiation. Type II pulmonary cells isolated from E19 embryos exhibited the greatest increase in strain-induced SP-C mRNA expression when cultured on laminin, as opposed to other ECM proteins. Interference with cell binding using soluble laminin peptides abrogated that response. In addition, blocking of integrins  $\alpha_6$  and  $\beta_1$  inhibited both cell adhesion and differentiation, whereas inhibition of  $\alpha_3$  prevented differentiation without altering cell attachment.

The ECM mediates the effects of mechanical stress on signal transduction and gene expression in numerous cell types (4, 5, 13, 27). The present investigations indicate that strain-induced differentiation of fetal type II cells is also modulated via specific cell-matrix interactions. Laminin plays an important

role in lung development and pulmonary epithelial cell differentiation and, in particular, may serve as an important regulator of tissue growth and pattern formation in the distal lung (32, 35, 39, 45, 46). Our data show that IKVIV, a synthetic peptide corresponding to the region near the globular portion of the long arm of the laminin  $\alpha$ -chain, and YIGSR, a laminin  $\beta$ -chain peptide, each inhibited stretch-induced SP-C expression without causing morphological changes or cell detachment. These findings indicate that laminin preferentially mediates strain-induced type II cell differentiation. This result is consistent with the finding that changes in cytoskeletal tensional forces that produce cell distortion also impact tissue growth and pattern formation in the distal regions of the embryonic lung (35). Although SP-C was maximally expressed when epithelial cells were strained on laminin substrates, other ECM proteins were also able to mediate, to different degrees, strain-induced type II cell differentiation. Despite the limitations of an *in vitro* system, these results suggest that, *in vivo*, several ECM proteins may contribute to fetal type II cell

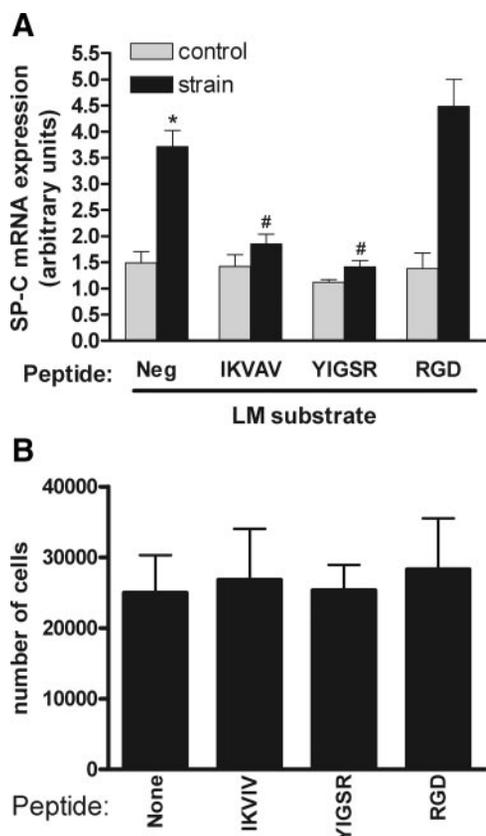


Fig. 3. Effects of laminin peptides on type II cell differentiation and adhesion. *A*: soluble peptides from the laminin  $\alpha$ -chain (IKVAV) and laminin  $\beta$ -chain (YIGSR) inhibit strain-induced SP-C mRNA expression (as measured in Northern blots) in cells cultured on laminin substrates, whereas addition of the cell-binding peptide from fibronectin (RGD) or no peptide (Neg) had no effect. Results are means  $\pm$  SE; \* $P$  < 0.001, # $P$  < 0.01. *B*: effects of soluble ECM peptides on cell attachment to laminin.

differentiation and underscore the complexity of ECM-cell interactions during fetal lung development.

In addition, our studies show that the level of activation of ERK by mechanical strain is not linearly related to the differentiation response of type II cells, at least as measured by SP-C expression. ERK was activated to a similar level by mechanical deformation of multiple ECM substrates, whereas levels of SP-C induction differed on these substrates. Other investigators have similarly observed that a JNK1 can be activated by application of mechanical strain to various ECM substrates in cardiac fibroblasts (31). The generalized activation of ERK by strain but restricted increase in SP-C expression indicates that other signals elicited by mechanical stress also contribute to the differentiation response. In addition, ERK phosphorylation mediated by different ECM-cell interactions in fetal lung development may not be limited to type II cell differentiation. In fact, strain-induced ERK activation influences cell proliferation in pulmonary epithelial H441 cells (6) and osmotic stress and apoptosis in adult type II cells (11). These studies suggest that strain-induced ERK activation, via ECM-cell interactions, may participate in a variety of cellular processes that are important for fetal lung development, in addition to differentiation.

Integrins are critical for lung organogenesis and maturation of the respiratory epithelium (10, 52, 58). However, the role of

integrins as mechanosensors in fetal lung development has not been explored. The main novel finding from our studies is the identification that specific integrin subtypes, including  $\beta_1$ ,  $\alpha_6$ , and  $\alpha_3$ , mediate lung differentiation in response to stress applied through cell adhesions to laminin. Interestingly, the different functional integrin subtypes also appeared to act via distinct mechanisms, including some that are independent of their cell-anchoring role.

$\beta_1$ -Integrin is abundantly expressed in type II cells and seems essential for the development of the lung epithelium (2), for cell anchoring to different substrates (9), and as mechanoreceptor in many other cell types (20, 29, 37, 44, 48, 51, 53). In agreement with these studies, cell adhesion experiments demonstrated that  $\beta_1$ -integrin mediates type II cell attachment to laminin substrates. Because any cell detachment caused by addition of these antibodies to adherent cell cultures would complicate the interpretation of our results, we then carried out experiments in which the flexible silastic membranes were coated directly with blocking anti- $\beta_1$ -integrin antibodies. In these studies, mechanical forces were applied to cells specifically through these bound receptors.  $\beta_1$ -Integrin specifically mediated strain-induced type II cell differentiation, as measured by SP-C expression. Antibodies that block substrate adhesion may activate transmembrane chemical signaling. This is consistent with past studies showing that application of mechanical stresses through nonactivating integrin antibodies can stimulate integrin-dependent signal transduction through the cAMP pathway (33). Other studies have also demonstrated that mechanical strain can induce chemical pathways that lead to integrin receptor activation (23, 24). Thus the key point here is that forces applied over these particular ligated integrins can trigger this cellular differentiation response. The signaling pathways triggered by mechanical stimulation of this receptor to induce lung differentiation are presently unknown. Theoretical mechanisms may include the recruitment of signaling proteins to focal adhesions via interaction with the actin cytoskeleton or activation of the ERK pathway via the adaptor protein Shc (5).

$\alpha_6$ -Integrin is another laminin binding receptor that mediates both cell attachment and differentiation in fetal type II cells. This is consistent with the finding that  $\alpha_6$ -integrin is upregulated in chondrosarcoma cells exposed to cyclic stretch (22).

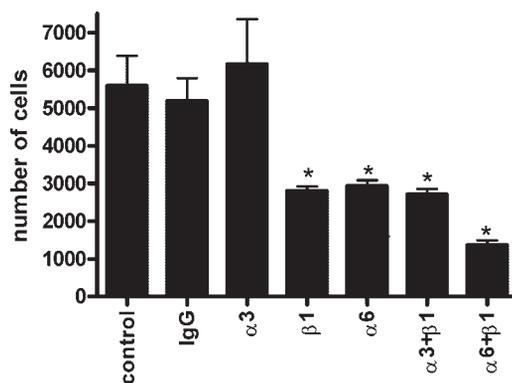


Fig. 4. Contribution of different integrin subtypes on cell adhesion to laminin. Cells were preincubated with antibodies against integrins  $\alpha_3$ ,  $\beta_1$ , or  $\alpha_6$ , alone or in combination, for 15 min at 37°C, and cultured on laminin substrates in the presence of antibody and cycloheximide for 4 h. Results are means  $\pm$  SE from 3 different experiments performed in triplicate (\* $P$  < 0.001).

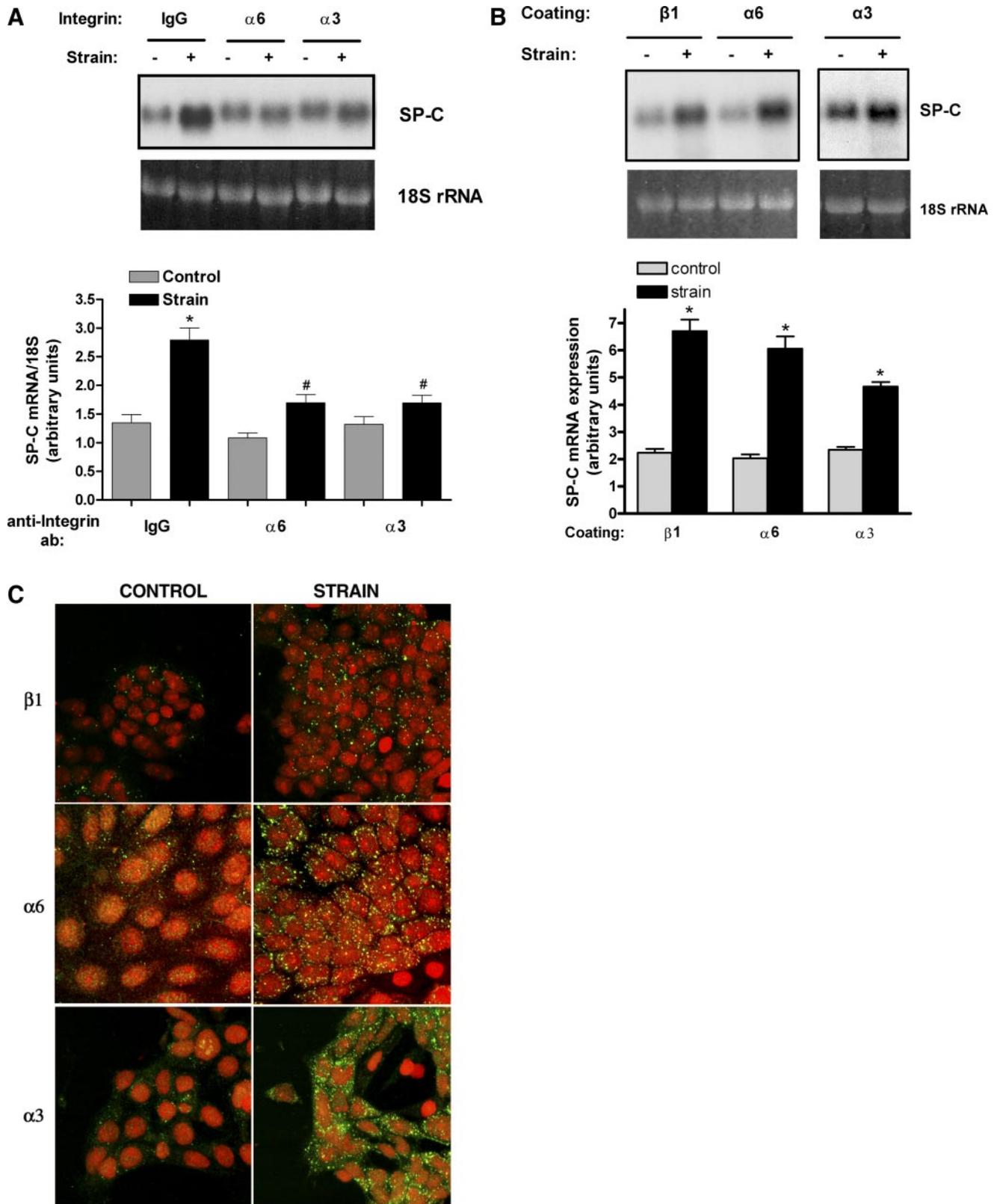


Fig. 5. Role of integrins in strain-induced differentiation. *A*: strain-induced SP-C mRNA expression is mediated by specific integrin subtypes. Shown is a representative Northern blot and graphical depiction of results from 3 separate experiments showing that antibodies against integrins α<sub>6</sub> and α<sub>3</sub> inhibit deformation-induced expression of SP-C in type II cells (\**P* < 0.001, #*P* < 0.01 relative to strain with IgG). *B*: representative Northern blot and results from 3 separate experiments showing strain induction of SP-C expression in cells cultured directly on substrates coated with anti-integrin β<sub>1</sub>, α<sub>6</sub>, and α<sub>3</sub> antibodies (\**P* < 0.05). *C*: representative confocal fluorescence microscopy images showing the effects of mechanical strain on SP-C protein levels (green) on substrates coated with anti-integrin β<sub>1</sub>, α<sub>6</sub>, and α<sub>3</sub> antibodies. Nuclei were counterstained with propidium iodide (red). Bar, 10 μm.

The  $\alpha_6\beta_1$  laminin receptor is also found at cell-ECM adhesions at sites of high stress due to cardiac contraction or blood flow induced by shear stress (16). It is presumed that  $\alpha_6\beta_1$ -integrin is critical for maintenance of architectural integrity and/or as mechanoreceptor in tissues constantly exposed to mechanical stresses.

To a lesser extent than  $\beta_1$  and  $\alpha_6$ ,  $\alpha_3$ -integrin was also identified to participate in strain-induced type II cell differentiation.  $\alpha_3\beta_1$ -Integrin regulates cytoskeletal organization in epithelial cells (54) and is critical for lung development (26). Expression of  $\alpha_1$ -,  $\alpha_2$ -, and  $\alpha_3$ -integrins also was greatly reduced or absent when Nkx2.1, a transcription factor essential for distal lung morphogenesis, was deleted (59). Of these receptors,  $\alpha_3$ -integrin modulates alveolar epithelial cell formation (30) and is abundantly expressed in epithelium during the canalicular stage of lung development (E19) (52), a time when mechanical forces exert maximal influence. Consistent with these observations, our findings suggest that  $\alpha_3$ -integrin may be important mechanosensor during late stages of fetal lung development. However, this integrin receptor was not critical for type II cell attachment to laminin substrates. In past studies,  $\alpha_V$  integrin was shown to mediate calcium signaling independently of its ability to mediate cell adhesion (47). Additionally, integrin binding alone is sufficient to induce signaling through other integrin receptors (17). Our studies agree with these observations and suggest that  $\alpha_3$ -integrin may facilitate transduction of mechanical signals into intracellular signaling responses necessary to trigger type II cell differentiation.

In summary, these studies demonstrate that strain-induced differentiation of fetal type II cells is mediated by specific ECM-integrin interactions. We have identified distinct laminin-binding integrin receptors that differentially mediate attachment to the substrate and participate in the differentiation of fetal lung epithelial cells. On the basis of the critical role played by mechanical forces in fetal lung development, our studies provide new insight into how these stresses influence distal lung epithelial cell differentiation. In particular, we demonstrate that specific integrin subtypes known to participate in the later stages of fetal lung development are activated by mechanical strain. These studies may facilitate development of new approaches to accelerate lung maturation in clinical conditions where lung development is impaired.

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