Laminin-Binding Integrin $\alpha 7$ Is Required for Contractile Phenotype Expression by Human Airway Myocytes

Thai Tran^{1,2,5}, Karen Ens-Blackie^{1,2,5}, Edward S. Rector³, Gerald L. Stelmack^{1,2,5}, Karol D. McNeill^{1,2,5}, Guido Tarone⁶, William T. Gerthoffer⁷, Helmut Unruh⁴, and Andrew J. Halayko^{1,2,4,5}

¹Departments of Physiology and Internal Medicine, ²CIHR National Training Program in Allergy and Asthma, ³Flow Cytometry Laboratory, and ⁴Sections of Respiratory Diseases and Thoracic Surgery, University of Manitoba, Winnipeg, Manitoba, Canada; ⁵Biology of Breathing Group, Manitoba Institute of Child Health, Winnipeg, Manitoba, Canada; ⁶Dipartimento di Genetica, Biologia e Biochimica, Molecular Biotechnology Center, Università di Torino, Torino, Italy; and ⁷Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada

Contractile airway smooth muscle (ASM) cells retain the ability for phenotype plasticity in response to multiple stimuli, which equips them with capacity to direct modeling and remodeling during development, and in disease states such as asthma. We have shown that endogenously expressed laminin is required for maturation of human ASM cells to a contractile phenotype, as occurs during ASM thickening in asthma. In this study, we profiled the expression of laminin-binding integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 7\beta 1$, and tested whether they are required for laminin-induced myocyte maturation. Immunoblotting revealed that myocyte maturation induced by prolonged serum withdrawal, which was marked by the accumulation of contractile phenotype marker protein desmin, was also associated with the accumulation of $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$. Flow cytometry revealed that $\alpha 7B$ expression was a distinct feature of individual myocytes that acquired a contractile phenotype. siRNA knockdown of $\alpha 7$, but not $\alpha 3$ or $\alpha 6$, suppressed myocyte maturation. Thus, $\alpha 7B$ is a novel marker of the contractile phenotype, and $\alpha 7$ expression is essential for human ASM cell maturation, which is a laminin-dependent process. These observations provide new insight into mechanisms that likely underpin normal development and remodeling associated with airways disease.

Keywords: airway remodeling; asthma; desmin; extracellular matrix; phenotype plasticity

During development and the pathogenesis of fibroproliferative disease, smooth muscle cells exhibit a dynamic phenotype in response to changes in biological cues such as growth factors and the extracellular matrix (ECM). This enables myocytes to act as determinants of acute and chronic pathologic features in diseases, such as airway remodeling in asthma. From studies with *in vitro* cell culture systems and *in vivo* animal models, it is well established that plastic phenotypic behavior of differentiated smooth muscle cells is marked by reversible modulation and maturation between contractile and proliferative/synthetic phenotypic states (1). Numerous ultrastructural, biochemical,

CLINICAL RELEVANCE

This study reveals new biological mechanisms relevant to fibroproliferative disorders such as asthma that are characterized by the accumulation of smooth muscle, and in which smooth muscle phenotype switching occurs.

and functional differences between phenotypic states, as well as numerous gene transcriptional and protein translation mechanisms that regulate phenotype expression, have been identified (2, 3). Among the external factors that can affect phenotype expression, the ECM plays a prominent role (4).

Laminin-2 is required for commitment of mesenchymal cells to the airway smooth muscle (ASM) lineage during lung development (5). *In vitro* studies with myocytes obtained from adult tissues show that although ECM proteins such as fibronectin and collagen I promote a proliferative phenotype (6), the laminin family of proteoglycans can suppress modulation of ASM cells from a contractile to proliferative phenotype (4). Moreover, we recently reported that maturation of human ASM from the proliferative to the contractile phenotype is associated with increased endogenous expression of the α , β , and γ laminin chains that constitute laminin-2 (7). Notably, using competing peptides for the integrin-binding YIGSR domain in these laminin chains, we further demonstrated that ASM binding to laminin-2 is essential for maturation of contractile phenotype myocytes enriched in protein markers such as desmin and calponin (7). This is of significance to understanding the pathogenesis of bronchial asthma, which is characterized by the concomitant deposition of ECM, including the laminin $\alpha 2$ chain (8), and a marked increase in contractile smooth muscle abundance in association with ASM hypertrophy. These observations strongly suggest the existence of a self-regulated biological mechanism, mediated through laminin-ASM interactions, that underpins key components of airway remodeling in asthma.

Although ECM constituents such as laminin are principal biological cues regulating phenotype plasticity of smooth muscle cells, relatively little is known about the repertoire of cell surface receptors needed to mediate their effects. The integrins are a large family of transmembrane proteins that exist as non-covalent heterodimers of α - and β -subunit splice variants that form receptors with different selectivity for individual ECM constituents (9). A specific group of laminin-binding integrins, including $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 7\beta 1$, has been identified (9). Glukhova and colleagues reported that vascular and colon smooth muscle cells exhibit concomitant changes in the spatial-temporal expression of laminin isoforms and laminin-binding integrins during development and maturation to adulthood (10). However, no studies have directly investigated the specific role of laminin-binding integrins in the maturation of differentiated smooth muscle cells to a contractile phenotype.

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Correspondence and requests for reprints should be addressed to Andrew J. Halayko, Section of Respiratory Disease, University of Manitoba, Room R5321, 810 Sherbrook Street, Winnipeg, MB, R3A 1R8 Canada. E-mail: ahalayko@cc.umanitoba.ca

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In the present study we characterized the repertoire of laminin-binding integrins expressed by adult human ASM cells, and tested the hypothesis that these receptors are required for maturation of myocytes to a contractile phenotype mediated by endogenously expressed laminin. With human ASM cell lines we used immunoblotting and real-time PCR to compare the expression of $\alpha 3$, $\alpha 6$, and $\alpha 7$ integrins and their splice variants in proliferating cultures, and cultures subjected to prolonged serum deprivation, which induces a subpopulation of human ASM cells to acquire the contractile phenotype (7, 11). Moreover, using flow cytometry and fluorescence microscopy we examined the unique repertoire of cell surface integrins expressed by human ASM cells of divergent phenotype. To test the requirement of specific integrins in the acquisition of a contractile phenotype, we employed selective siRNAs to silence expression of individual integrins and assessed the effect on expression of stringent phenotype markers. Collectively, these studies demonstrate for the first time that the laminin-binding $\alpha 7$ integrin subunit, most likely $\alpha 7 \beta 1$, is exclusively needed to promote contractile phenotype acquisition in differentiated human ASM cells.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade or higher. All compounds were purchased from Sigma (St. Louis, MO) unless stated otherwise. Rabbit anti-desmin antibody (H-76, sc14026) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies directed against integrins $\alpha 3 A$ (clone 29A3), $\alpha 3 B$ (clone 54B3), $\alpha 5$, $\alpha 6 A$ (clone 1A10), $\alpha 6 B$ (clone 6B4), and $\beta 1$ (clone Lia1/2) were purchased from Chemicon (Temecula, CA). Antibodies directed against integrins $\alpha 7 A$ and $\alpha 7 B$ were gifts from Dr. G. Tarone, Università di Torino, Italy (12).

Immortalized Human ASM Cell Culture

For all studies senescence-resistant human ASM cell lines were generated using stable MMLV retroviral transduction of the human telomerase reverse transcriptase gene (hTERT) into passage 1 or 2 primary cultures as we have described (7, 13). Primary cultured human ASM cells were prepared from macroscopically healthy segments of second- to fourth-generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (7, 13). All procedures were approved by the Human Research Ethics Board (University of Manitoba). hTERT-expressing human ASM cells retain the ability to express markers of the contractile phenotype including smooth muscle myosin heavy chain (smMHC), calponin, sm- α -actin, and desmin to passage 10 and higher (13). At least four different hTERT-human ASM cell lines between passages 16 and 22 were used in all studies.

Unless otherwise indicated, human ASM cells were seeded at a density of 1×10^4 cells/cm², and grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% vol/vol fetal bovine serum (FBS) (14). To induce a contractile phenotype in hTERT-human ASM, confluent cultures were switched to DMEM supplemented with ITS (insulin 5 μ g/ml; transferrin 5 μ g/ml, selenium 5 ng/ml) for up to 7 days as we have described (3, 7, 13, 14). Serum-free media was replaced every 3 days. With this protocol, increased expression of desmin and sm-

α -actin was consistently induced, indicative of phenotype maturation in both hTERT-human ASM and primary cultured human ASM cells.

Measurement of Protein Abundance

For analysis of myocyte phenotype, protein lysates were prepared as we have described (7) in ice-cold lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 7.5; 2 mM EDTA; 0.5% wt/vol deoxycholate; 1% vol/vol triton X-100; 1 mM phenylmethylsulfonylfluoride; 10 mM MgCl₂; 5 μ g/ml aprotinin; 100 μ M sodium orthovanadate). Soluble protein content was determined using the Bio-Rad protein assay (BioRad, Hercules, CA). For measuring integrin protein abundance, samples were size fractionated by SDS-PAGE using loading buffer lacking β -mercaptoethanol (β -MC). For all other proteins β -MC was included in the gel loading buffer. Western blotting was performed as we have described using nitrocellulose membranes (7). Optimal dilutions for each antibody were pre-determined: anti-desmin (1:500), anti-sm- α -actin (1:1,000), anti- $\alpha 3 A$ (1:1,000), anti- $\alpha 5$ (1:5,000), anti- $\alpha 6 A$ (1:500), anti- $\alpha 7 B$ (1:1,000), and anti- $\beta 1$ (1:500). After protein transfer membranes were developed using secondary horseradish peroxidase-conjugated antibody, and visualized by enhanced chemiluminescence reagents (Amersham, Oakville, ON, Canada). Membranes were re-probed with antibodies for β -actin (Sigma) to normalize for equal sample loading. Densitometry was performed using the Epson Perfection 4180 Station and TotalLab TL100 software (Nonlinear Dynamics, Durham, NC).

Measurement of mRNA Abundance

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. RNA (2 μ g) was reversed transcribed using M-MLV reverse transcriptase (Promega, Madison, WI), incubated for 2 hours at 37°C followed by 5 minutes of incubation at 95°C, and diluted 1:10 with RNase-free water. Real-Time PCR was performed using the resulting cDNA with primer pairs listed in Table 1. Reaction parameters were: 40 cycles consisting of 92°C for 45 seconds (denaturation), 60°C for 45 seconds (annealing), and 72°C for 90 seconds (extension). Assays were performed in duplicate in 20- μ l reactions, and the cycle threshold (C_T = amplification cycle number) values for each reaction were determined using the Roche Molecular Biochemicals LightCycler 3 (version 3.5; Indianapolis, IN). Data were analyzed using the comparative C_T method as previously described (7) using the Applied Biosystems (Streetsville, ON, Canada) guidelines (15). The fold change in gene expression normalized to an endogenous reference gene (18 s rRNA) and relative to the untreated control (Day 0) is given by the equation $2^{-\Delta\Delta C_T}$. The ΔC_T value is determined by subtracting the average 18 s rRNA C_T value from the average C_T value of the corresponding target gene of interest. The calculation of $\Delta\Delta C_T$ values involves subtraction by the ΔC_T calibrator value (Day 0). For the untreated sample (Day 0) $\Delta\Delta C_T = 0$ and 2^0 equals 1. For the treated samples, evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold change in gene expression relative to Day 0.

Flow Cytometry

Our previous studies show human ASM is a heterogeneous population in which $\sim 1/6$ th of cells are induced to a fully contractile phenotype with prolonged serum deprivation (3, 14). Therefore, we used flow cytometry to directly assess co-expression of cell surface integrin subunits and the intracellular intermediate filament protein desmin, a stringent contractile phenotype marker, in individual myocytes from

TABLE 1. PRIMERS USED FOR REAL-TIME PCR TO ASSESS INTEGRIN EXPRESSION

Gene Product	NCBI Accession No.	Primer Sequences	PCR Product Size
Integrin $\alpha 3 A$	NM002204	Forward 5' CAG AGA GAC CCT GCA AGA CC 3'	200 bp
		Reverse 5' CTC AAA TGG GCC TTA GGA CA 3'	
Integrin $\alpha 6 A$	NM000210	Forward 5' GGA GCC CCA CAG TAT TTT GA 3'	244 bp
		Reverse 5' TTC CAT TTG CAG ATC CAT GA 3'	
Integrin $\alpha 7 B$	NM002206	Forward 5' ACC AAT ACC CTG ACC TGC TG 3'	202 bp
		Reverse 5' CTA TAG CTG CTG GGG ACT GC 3'	
18s rRNA	(7)	Forward 5' 5'CGC CGC TAG AGG TGA AAT TC 3'	92 bp
		Reverse 5' TTG GCA AAT GCT TTC GCT C 3'	

cultures maintained for 7 days in serum-free DMEM/ITS media. Cells were detached from dishes using 0.025% vol/vol trypsin/10 mM EDTA, and rapidly transferred to a large volume of PBS containing 0.2% vol/vol FBS to quench trypsin activity (16). Cell suspensions were centrifuged ($800 \times g$, 5 min), resuspended in 10 ml PBS, filtered through 70- μ m nylon mesh, and cell number was determined using a hemocytometer. Aliquots containing 10^6 intact cells were incubated (30 min, room temperature) with surface-staining integrin antibodies (1 μ g/100 μ l) that had been pre-conjugated with Alexa-Fluor (AF)-488 using Zenon Labeling Kit (3.5:1 molar ratio of Zenon labeling reagent to antibody) according to manufacturer's instructions (Molecular Probes, Invitrogen, Eugene, OR). After incubation with Zenon AF-488-anti-integrin IgG, human ASM cells were fixed in 3% paraformaldehyde/PBS (5 min, room temperature), washed three times with PBS, and then permeabilized in PBS/0.1% Triton X-100 (5 min, room temperature). Cells were then centrifuged ($800 \times g$, 5 min), washed once with 1 ml PBS, and then resuspended and incubated (30 min, room temperature) in rabbit anti-desmin IgG₁ (1 μ g/100 μ l) which had been pre-conjugated with the R-Phycoerythrin (R-PE) using the Zenon Labeling Kit. Cells were then washed and resuspended in PBS in preparation for flow cytometry. For all experiments background and nonspecific labeling was determined using control cells incubated with isotype-matched nonimmune serum that had been conjugated with either AF-488 or R-PE using protocols that matched those used for primary antibodies.

Flow cytometry was performed using a Beckman Coulter EPICS ALTRA Flow Cytometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada). All analyses used 488-nm (150 mW) laser excitation. Forward light and side scatter histograms were used to identify and gate for intact cells. AF-488 and R-PE emission was collected simultaneously using 525-nm and 575-nm bandpass filters, respectively. For each histogram 10,000-gated events were collected. Labeling for integrins or desmin was considered "positive" when fluorescence intensity was greater than that observed for all but the highest 5% of cells stained with isotype-matched antibodies. EXPO32 MultiCOMP MFA software (Version 1.2B; Beckman Coulter Canada) was used to assess each myocyte for the percent overlap in coincident positive and negative staining for cell surface integrins and desmin.

Immunocytochemistry

hTERT-human ASM cells were grown to confluence on 25 mm² microscope glass slides, subjected to 7-day serum-free culture in DMEM/ITS, then were fixed, permeabilized, and immunolabeled as we have previously described (3, 14). Human bronchial specimens obtained from lung resection were quick-frozen in OCT compound and then ~ 7 - μ m cross-sections were cut. Sections were mounted on microscope slides, then fixed and permeabilized and labeled with antibodies as described for cultured cells. Primary antibodies and their respective dilutions included rabbit anti-desmin IgG (1:100) alone, and in combination with either mouse anti-smMHC IgG₁ (clone hSMv; 1:100; Sigma ImmunoResearch) or rabbit-anti-integrin $\alpha 7$ B IgG₁ (1:50) (12). Fluorescein isothiocyanate- and Texas Red-conjugated secondary antibodies (1:100; Jackson ImmunoResearch, West Grove, PA) were used to detect primary antibody bound to labeled cells. Nuclei were then labeled with Hoechst 3342 (10 μ g/ml), and coverslips were mounted using ProLong Antifade (Molecular Probes, Invitrogen). Thereafter fluorescence micrographs were obtained as we have described (3) using an Olympus LX70 microscope equipped with charge coupled camera controlled by UltraView Software (Olympus, Hicksville, NY).

siRNA Preparation and Study Design

The small interfering RNA (siRNA) Generation Kit (Gene Therapy Systems, San Diego, CA) was used to prepare siRNA from human ASM cDNA with primers that amplified integrin $\alpha 3$ cDNA (558 base pairs [bp]), $\alpha 6$ cDNA (523 bp) and $\alpha 7$ cDNA (547 bp, Table 2). PCR primers were designed to amplify unique regions of each specific integrin that did not share sequence homology with any other integrins. Primer sequences listed in Table 2 also included the 52-bp T7 promoter sequence linkers (5'-GCGTAATACGACTCACTATAGGAGA-target DNA-3'), which were incorporated into the DNA template PCR product to allow for *in vitro* transcription with the TurboScript T7 Transcription Kit (Gene Therapy Systems). Double-stranded RNA (dsRNA) was generated

TABLE 2. PRIMERS USED TO GENERATE SMALL INTERFERING RNA FROM HUMAN AIRWAY SMOOTH MUSCLE CELL cDNA

Gene Product	NCBI Accession No.	Primer Sequences	PCR Product Size*
Integrin $\alpha 3$	NM002204	Forward 5' GATGACTGTGAGCGGATG 3' Reverse 5' CTGGCTCAGCAAGAACAC 3'	610 bp
Integrin $\alpha 6$	NM000210	Forward 5' GTGTTATGTCCTGAGTCAG 3' Reverse 5' CTTGTTGAGGTCCACCAC 3'	575 bp
Integrin $\alpha 7$	NM002206	Forward 5' GACCAGATCCTGGAGACG 3' Reverse 5' CATCACTGTTGAGGTCAGC 3'	599 bp

* Includes the 52 bp T7 promoter sequence linkers mentioned in MATERIALS AND METHODS.

using the TurboScript T7 RNA Transcription Kit and then diced into 21-bp fragments using recombinant human dicer enzyme following the manufacturer's instructions (Gene Therapy Systems).

In preliminary experiments, from a concentration-response curve (0.05–1 μ g/ml) we determined that 1 μ g/ml of siRNA provided optimal silencing of mRNA and protein for all integrin targets ($\alpha 3$, $\alpha 6$, $\alpha 7$) in hTERT-human ASM cells. For subsequent studies the day before siRNA transfection, confluent human ASM cells were re-seeded into 6-well plates in DMEM/10% vol/vol FBS so that 50 to 60% confluence was achieved 16 hours later. Transfection of siRNA (1 μ g/ml) was performed with Genesilencer reagent (Gene Therapy Systems) according to manufacturer's instructions, and cells were maintained in serum-free DMEM/ITS thereafter. A second transfection was performed 3 days later at the same time that culture media was refreshed. This double transfection strategy was developed based on preliminary experiments, which showed it was most effective at silencing integrin mRNA and protein expression for at least 6 days. Transfection efficiency at each stage was greater than 95%, as we have previously reported (13). For negative control studies the transfection protocol was performed in the absence of siRNA.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. All experiments were completed in duplicate using hTERT-human ASM generated from at least four different primary bronchial smooth muscle cell cultures. Protein expression data is expressed as fold change compared with initial levels (Day 0 in most experiments); all values are corrected for equal loading based on β -actin abundance. Comparisons were made using one-way ANOVA, with repeated measures, followed by Bonferroni's *post hoc t* test. A probability value of $P < 0.05$ was considered significant.

RESULTS

Laminin-Binding Integrin Expression Is Increased during Human ASM Maturation

We have shown that maturation of hTERT-human ASM cells to a contractile phenotype during prolonged serum-free culture is marked by laminin-2 dependent accumulation of contractile phenotype marker proteins such as desmin, calponin, and smooth muscle α -actin (7, 13). Indeed, concomitant with myocyte maturation the cells exhibit increased expression of the $\alpha 2$, $\beta 1$, and $\gamma 1$ chains of laminin-2. Thus, we assessed whether human ASM cell maturation was also associated with changes in expression of laminin-binding integrin subunits $\alpha 3$, $\alpha 6$, and $\alpha 7$, and their respective A and B cytoplasmic tail splice variants.

Using immunoblotting we observed that the abundance of integrins $\alpha 3$ A, $\alpha 6$ A, and $\alpha 7$ B were approximately doubled (Figure 1) in culture conditions that promoted the maturation of human ASM cells to a contractile phenotype (7). In contrast, integrin $\alpha 3$ B (1.0 ± 0.02) and $\alpha 7$ A (1.0 ± 0.02) abundance was unchanged during myocyte maturation. Moreover, neither the fibronectin selective integrin $\alpha 5$ subunit (17) nor integrin $\beta 1$ were changed in abundance with serum deprivation (Figure 1). To

Figure 1. (A) Western blot and (B) real-time PCR analysis showing the protein and mRNA expression, respectively, of selective variants of laminin-binding integrins before (Day 0, open bars) and after 7-day serum deprivation (Day 7, solid bars). Grouped data, which are expressed as relative units to Day 0, represent results obtained from at least four different cultures. * $P < 0.05$, compared with Day 0, relative to β -actin. (C) PCR analysis showing expression of laminin-binding integrins and their respective A and B cytoplasmic splice variants in airway smooth muscle (ASM) cells dissected from human bronchial tissue.

further confirm these results we employed quantitative real-time PCR analyses, which revealed a 3- to 8-fold increase in mRNA for integrins $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$ over the course of myocyte maturation (Figure 1). These experiments reaffirmed that there was no change in expression of $\alpha 3B$, $\alpha 7A$, $\alpha 5$, and $\beta 1$ subunits, and also demonstrated that expression of $\alpha 6B$, for which we had no suitable antibodies to perform immunoblotting, was similarly unchanged during myocyte maturation (not shown). Collectively these experiments reveal for the first time that increased expression of laminin-binding integrin subunits $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$ occurs in culture conditions that promote the maturation of human ASM cells to a contractile phenotype.

In additional experiments we performed PCR analyses to determine whether the profile of laminin-binding integrins expressed by contractile human ASM in prolonged serum-free culture mimicked that of myocytes from intact tissue. We designed primers that generated PCR products of differing size for each of the A and B splice variants of $\alpha 3$, $\alpha 6$, and $\alpha 7$ integrins (Table 3). Using mRNA isolated from human ASM cells dissected from second-generation mainstem human bronchi, we observed that $\alpha 3B$, $\alpha 6A$, and $\alpha 7B$ isoforms were highly expressed, $\alpha 3A$ and $\alpha 6B$ transcript was present in low abundance, and $\alpha 7A$ was undetectable (Figure 1).

Surface Expression of Laminin-Binding Integrins on Human ASM

Though our immunoblotting and PCR analyses confirm increased expression of laminin-binding integrins in conditions that pro-

mote maturation, our previous studies have revealed there is a disparate induction of the contractile phenotype marker proteins in distinct subpopulations of cultured airway myocytes under these conditions (3, 14). Thus, we used flow cytometry to assess cell surface abundance of integrins in individual myocytes after 7 days of serum-free culture. Figure 2 shows representative frequency distribution histograms of human ASM cells that were surface stained for laminin-binding integrin subunits using fluorochrome-conjugated primary antibodies. To confirm the threshold for positive labeling, for all experiments we prepared parallel negative control samples using fluorochrome-conjugated isotype-matched immunoglobulin. Distinct fractions of cells exhibiting positive fluorescence labeling for integrins $\alpha 3A$ ($46 \pm 16\%$), $\alpha 6A$ ($42 \pm 19\%$), $\alpha 6B$ ($27 \pm 19\%$), and $\alpha 7B$ ($16 \pm 6\%$) were observed (Figure 2). In contrast, though a small fraction of cells labeled for $\alpha 3B$ or $\alpha 7A$ was evident, these myocytes appeared as part of a shoulder from the larger population of negatively stained myocytes. As a positive control for cell surface integrin labeling, we also performed experiments to assess $\alpha 5$ and $\beta 1$ integrin expression (Figure 2). Notably, compared with respective isotype controls, a distinct positively stained peak that included virtually all myocytes was evident for both $\alpha 5$ and $\beta 1$. Collectively, in a pattern mimicking our previous investigation of contractile phenotype marker protein expression (3, 14), the current experiments show that disparate expression of cell surface laminin-binding α -integrin subunits is induced in subpopulations of human airway myocytes when cells are grown in conditions that promote contractile phenotype maturation.

TABLE 3. PRIMERS USED FOR PCR TO ASSESS INTEGRIN EXPRESSION IN AIRWAY SMOOTH MUSCLE CELLS DISSECTED FROM HUMAN BRONCHIAL TISSUE

Gene Product	NCBI Accession No.	Primer Sequences	PCR Product Size
Integrin $\alpha 3$	J05281	Forward 5' AAG CCA AAT CTG AGA CTG TG 3'	($\alpha 3A$) 650 bp
		Reverse 5' GTA GTA TCG GTC CGA ATC T 3'	($\alpha 3B$) 500 bp
Integrin $\alpha 6$	BC058095	Forward 5' GTC AGG TGT GTG AAC ATC AG 3'	($\alpha 6A$) 540 bp
		Reverse 5' CAT GGT ATC GGG GAA TGC T 3'	($\alpha 6B$) 410 bp
Integrin $\alpha 7$	NM008398	Forward 5' GTT GTG GAA GGA GTC CC 3'	($\alpha 7A$) 280 bp
		Reverse 5' GTC TTC CCG AGG GAT CTT 3'	($\alpha 7B$) 170 bp

Figure 2. Flow cytometry analysis of surface expression of laminin-binding integrins in human ASM cells serum deprived for 7 days. Each histogram contains data obtained from 10,000 gated cells. Representative frequency histograms showing the distribution of myocytes stained for integrin (A) $\alpha 3A$, (B) $\alpha 3B$, (C) $\alpha 6A$, (D) $\alpha 6B$, (E) $\alpha 7A$, (F) $\alpha 7B$, (G) $\alpha 5$, or (H) $\beta 1$. The gray lines in each panel show the distribution of corresponding negative controls that included myocytes incubated in diluted mouse IgG1 (A–D), mouse IgG2a (G), or rabbit (E and F).

Co-Expression of Laminin-Binding Integrins and Contractile Phenotype Markers

We next used flow cytometry to investigate whether there was a direct association between induction of laminin-binding α -integrin subunits and the acquisition of a contractile phenotype in individual myocytes. For this purpose we developed double labeling protocols to assess the expression of membrane integrins and intracellular desmin, an intermediate filament protein that is a stringent marker of the contractile phenotype in smooth muscle cells (11). No direct correlation between the expression of integrin $\alpha 3A$ and desmin was seen (Figure 3), despite our previous observation that both proteins accumulate significantly during contractile phenotype development (Figure 1). Indeed, only about half ($43 \pm 2\%$) of the myocytes that expressed $\alpha 3A$ also expressed desmin. Similarly, there was no correlation between $\alpha 6A$ and desmin, with co-expression in only half ($43 \pm 2\%$) of $\alpha 6A$ -positive myocytes (Figure 3). Labeling for integrins $\alpha 3B$, $\alpha 6B$, or $\alpha 7A$, which were unchanged in total abundance

during 7-day serum deprivation, was also not associated with an increased tendency for expression of desmin (Table 4). In contrast, labeling for integrin $\alpha 7B$ exhibited a significant positive correlation with desmin expression in individual human ASM cells, as $66 \pm 3\%$ of $\alpha 7B$ -positive myocytes co-expressed desmin ($P < 0.05$, ANOVA with repeated measures, and Bonferroni's *post hoc* test, Figure 3). These data indicate that though integrins $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$ each accumulate in total abundance in human ASM cell culture during prolonged serum deprivation (Figure 1), only the $\alpha 7B$ subunit appears to be selectively induced in the unique subpopulation of myocytes that acquires a contractile phenotype (Figure 3). This finding was consistent in each of the three independent experiments that we performed.

To further confirm the selective induction of integrin $\alpha 7B$ in human ASM cells expressing a contractile phenotype, we performed immunofluorescence imaging of primary cultured human ASM cells and intact bronchial tissue (Figure 4). Primary human ASM cells subjected to 7-day serum deprivation were double

Figure 3. Dual fluorescence analysis of integrin subunit and desmin expression in 7-day serum-deprived human ASM cells. (A) Representative two-parameter histogram dot plot showing the isotype controls for Zenon Alexa-Fluor 488 (AF-488) and Zenon R-PE (R-PE). Representative two-parameter histogram dot plots showing distribution of fluorescence staining of myocytes with AF-488 conjugated primary antibodies for integrins $\alpha 3A$ (B), $\alpha 6A$ (D), or $\alpha 7B$ (F) and R-PE-conjugated primary antibody for desmin are shown. Histograms showing the distribution of positive (+) and negative (-) staining for desmin in myocytes gated for (+) or (-) labeling of integrins $\alpha 3A$ (C), $\alpha 6A$ (E), or $\alpha 7B$ (G). Grouped data from three replicate two-parameter histogram dot plots are shown.

labeled for $\alpha 7B$ and either desmin or smooth muscle myosin heavy chain (smMHC), two stringent protein markers of the contractile phenotype that show strong coincident staining in contractile phenotype myocytes (Figure 4). Notably, as expected from our prior flow cytometry analyses, we observed abundant expression of $\alpha 7B$ in a unique subpopulation of human ASM cells, and this appeared to be entirely coincident with desmin and smMHC labeling. Moreover, these cells exhibited a large elongate morphology that is characteristic of contractile phenotype smooth muscle cells, and expression of both smMHC and desmin was a unique feature of these cells (Figure 4). We also performed immunofluorescence imaging of intact mainstem human airway specimens, and consistently observed strong labeling for integrin $\alpha 7B$ in the contractile smooth muscle layer surrounding the

bronchi (Figure 4). Collectively these data confirm a strong correlation for abundant expression of $\alpha 7B$ integrin in ASM cells of a contractile phenotype.

Requirement of $\alpha 7$ Integrin for Human ASM Maturation: siRNA Protocols

Based on our current flow cytometry and immunofluorescence studies, our previous experiments showing that endogenous laminin, likely laminin-2, is required for human ASM cell maturation (7), and that integrin $\alpha 7$ selectively binds laminin-2/4 (18), we next assessed the requirement for integrin $\alpha 7$ in human ASM cell maturation using siRNA technology. Human ASM cells were seeded at confluence and maintained in serum-free conditions for 6 days, during which time cells were transfected on both Day 0 and

TABLE 4. RESULTS FROM FLOW CYTOMETRY TO ASSESS CO-EXPRESSION OF DESMIN AND INTEGRIN ISOFORMS, $\alpha 3B$, $\alpha 6B$, AND $\alpha 7A$

	Integrin Gating (+)		Integrin Gating (–)	
	Desmin (–)	Desmin (+)	Desmin (–)	Desmin (+)
Integrin $\alpha 3B$	56.5 $\pm$ 1.5	43.5 $\pm$ 1.5	65.5 $\pm$ 5.5	34.5 $\pm$ 5.5
Integrin $\alpha 6B$	57.0 $\pm$ 2.0	43.0 $\pm$ 2.0	62.5 $\pm$ 12.5	37.5 $\pm$ 12.5
Integrin $\alpha 7A$	61.5 $\pm$ 2.5	39.0 $\pm$ 3.0	66.0 $\pm$ 7.0	34.0 $\pm$ 7.0

Data are shown as percent (%) positive (+) or negative (–) staining for desmin in myocyte subpopulations gated for + or – integrin labeling.

Day 3, with siRNA selective for integrin $\alpha 7$ (Table 2). We first assessed the effectiveness of siRNA-mediated silencing of integrin $\alpha 7$ expression during 6-day serum deprivation using PCR and immunoblotting for $\alpha 7B$ (Figure 5). Both analyses revealed that the siRNA protocol we developed completely abrogated the accumulation of integrin $\alpha 7B$ that typically occurs in human ASM cells, and was also evident in our control samples.

We next examined the effects of inhibiting $\alpha 7$ integrin accumulation with siRNA on human ASM cell maturation by measuring the accumulation of the contractile phenotype marker proteins desmin and sm- α -actin (7, 11, 14). Consistent with phenotype maturation of ASM cell myocytes that we have described previously (7, 13, 14), before serum deprivation human ASM cells expressed low levels of desmin and sm- α -actin (Figure 5), but these levels were approximately doubled after 6 days of growth in serum-deficient conditions. Notably, with inhibition of integrin $\alpha 7$ expression using siRNA, accumulation of both desmin and sm- α -actin was completely prevented (Figure 5), suggesting an abolition of phenotype maturation. Importantly, accumulation of desmin and sm- α -actin after 6 days in control transfection samples was the same as that seen in untreated cultures. For example, in untreated samples and control transfection samples, desmin increased 1.8 ± 0.2 and 1.9 ± 0.3 times, respectively ($P > 0.05$), compared with Day 0.

To confirm the validity of our findings and a possible unique functional role for $\alpha 7$ integrin in myocyte maturation, we next performed experiments to ensure that our siRNA protocol targeting $\alpha 7$ integrin was selective, and that there were no indirect effects on expression of other integrin subunits. Indeed, silencing of integrin $\alpha 7$ had no concomitant effects on $\alpha 3A$ and $\alpha 6A$ (Figures 6A and 6B), the $\alpha 5$ subunit (Figure 6C), or on integrin $\beta 1$ (Figure 6D). These findings strongly implicate a unique requirement for $\alpha 7$ integrin in phenotype maturation of human ASM cells from the adult lung.

To ensure that the ability of silencing $\alpha 7$ integrin to prevent myocyte phenotype maturation was not due to cell toxicity, we assessed cell integrity by phase contrast imaging (Figures 6E–6H), and measured cell number after completing the siRNA protocol. There was no evidence of myocyte stress, nor were there any differences in the total number of adherent cells after the 6 days of siRNA treatment ($1.0 \pm 0.1 \times 10^5$ cells/well versus $1.0 \pm 0.1 \times 10^5$ cells/well in control treated and $\alpha 7$ siRNA-treated respectively; $P > 0.05$).

Unique Requirement of $\alpha 7$ Integrin in Phenotype Maturation of Human ASM Cells

Though our data using selective $\alpha 7$ integrin silencing reveal a requirement for this receptor in phenotype maturation, they do not preclude the possibility that other laminin-binding integrins may also be involved. Thus we next developed siRNA protocols for integrins $\alpha 3$ and $\alpha 6$ and measured the effects of silencing these subunits on myocyte phenotype expression in

Figure 4. Matched immunofluorescence images of primary human ASM cells and intact human bronchial tissue double-labeled for integrin $\alpha 7B$ and desmin or smMHC. High magnification fields showing typical co-expression of integrin $\alpha 7B$ (A) with desmin (B), and $\alpha 7B$ (C) with smMHC (D), after prolonged serum withdrawal of primary cultured human ASM cells. Low-power images show coincident expression of the contractile phenotype marker proteins desmin (E) and smMHC (F). Representative immunofluorescence imaging of integrin $\alpha 7B$ in smooth muscle layer from 3rd generation human mainstem bronchus is shown in G. Labeling of bronchial tissue with isotype-matched control antibody and fluorescein isothiocyanate-conjugated secondary antibody is shown in H. Arrows indicate matching cells in A and B, C and D, and E and F, respectively. Abbreviations: EPI, epithelium; SM, airway smooth muscle. Bar = 50 μ m.

serum-free culture conditions. In a manner similar to our studies using $\alpha 7$ siRNA, confluent cultures of human ASM cells were maintained for 6 days in serum-free culture, during which time cells were transfected with selective siRNA for $\alpha 3$ or $\alpha 6$ integrin (Table 2) on Day 0 and Day 3. To confirm the effectiveness of $\alpha 3A$ and $\alpha 6A$ silencing, we measured the abundance of each protein after 6 days of serum deprivation in the presence of the respective siRNA we generated (Figure 7). Importantly, the increase in $\alpha 3$ or $\alpha 6$ integrin that we observed in control conditions (Figure 1) was abrogated by their corresponding siRNA (Figure 7).

Figure 5. Analysis of the effect of siRNA-induced suppression of integrin $\alpha 7$ mRNA and protein over 6 days of serum-free culture. (A) PCR analysis; $\alpha 7$ PCR product size = 599 base pairs (bp). (B) Western blotting for integrin $\alpha 7B$. Also shown is Western blot analysis of the effects of $\alpha 7$ siRNA on desmin and smooth muscle α -actin accumulation after 6 days of serum deprivation. Grouped data, which are expressed as relative units to Day 0, represent results obtained from at least four different cultures. * $P < 0.05$, compared with Day 0, relative to β -actin; † $P < 0.05$, compared with control cultures (6-day serum-free media exposed to transfection reagent without siRNA).

Silencing of integrin $\alpha 3$ was selective, as $\alpha 3$ siRNA was without effect on the accumulation of integrin $\alpha 6A$ and $\alpha 7B$ during prolonged serum withdrawal (Figure 7, Table 5). Integrin $\alpha 3$ silencing did, however, promote protein accumulation of the fibronectin-binding receptor, integrin $\alpha 5$, during 6-day serum-free conditions, a response that was mimicked by siRNA for $\alpha 6$ integrin, and that was not seen in control conditions (Table 5). Nonetheless, in contrast to results we obtained with $\alpha 7$ siRNA (Figure 5), silencing of $\alpha 3$ failed to prevent myocyte phenotype maturation, as the accumulation of desmin and sm- α -actin was similar in control and $\alpha 3$ siRNA treated cultures (Figure 8). Collectively, these data indicate that our previous observation of a unique requirement for increased $\alpha 7$ integrin expression for human ASM cell phenotype maturation is not dependent on a concomitant increase in $\alpha 3$ integrin expression.

In the design of primers to generate integrin-selective siRNAs, we chose coding regions that had poor homology between integrins (Table 2). Despite these efforts, we found that treatment with siRNA we generated for $\alpha 6$ integrin blocked accumulation of both $\alpha 6A$ and $\alpha 3A$ integrin proteins, and thus had a double gene-silencing effect (Figure 7). Importantly, however, consistent with our results using siRNA for $\alpha 3$ integrin, integrin $\alpha 6$ siRNA treatment did not affect the accumulation of integrin $\alpha 7B$ that occurs over 6 days of serum deprivation (Table 5). With respect to human ASM cell phenotype maturation, in marked contrast to the results we obtained with selective $\alpha 7$ silencing (Figure 5), treatment with $\alpha 6$ siRNA, and subsequent silencing of both $\alpha 6$ and $\alpha 3$, was without effect on the accumulation of desmin and sm- α -actin during 6 days of serum withdrawal (Figure 8). These data further strengthen our observations that suggest a unique requirement of laminin-binding $\alpha 7$ integrin in human ASM cell

phenotype maturation, as concomitant suppression of $\alpha 6$ and $\alpha 3$ integrin expression failed to prevent acquisition of a contractile phenotype *in vitro*.

DISCUSSION

Phenotype plasticity of smooth muscle cells in differentiated tissues is a key determinant of organ remodeling associated with fibroproliferative disorders such as asthma. In this regard, primary focus has been given to modulation of myocytes to a proliferative/synthetic state. Thus, though it underpins increased smooth muscle mass that leads to altered vascular and lung function, less is known about the mechanisms promoting myocyte maturation. Intracellular markers of mature smooth muscle cells have been described; however, the current study is the first to show that increased expression of a select repertoire of integrins that bind laminin is both a hallmark of the contractile phenotype, and is required for the process of phenotype maturation. Our studies demonstrate that expression of the laminin-binding integrins, $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$, is significantly increased in human airway myocytes under conditions that promote phenotype maturation. Notably, with prolonged serum-free culture, distinct subpopulations of human ASM cells are revealed; using dual immunofluorescence flow cytometry protocols, we demonstrate that among the laminin-binding integrins, only $\alpha 7B$ integrin is preferentially expressed by the individual myocytes in the subgroup that acquires a contractile phenotype. Moreover, using gene-silencing strategies with integrin-selective siRNAs, we show that $\alpha 7$ integrin (likely $\alpha 7B$), but not $\alpha 3$ and $\alpha 6$, mediates laminin-dependent myocyte maturation. Our observations are significant because they reveal intrinsic ECM-associated mechanisms

Figure 6. Western blot analysis showing the effect of siRNA silencing of integrin $\alpha 7$ on the accumulation of integrins $\alpha 3A$ (A), $\alpha 6A$ (B), $\alpha 5$ (C), and $\beta 1$ (D) over 6 days of serum deprivation. Grouped data, which are expressed as relative units to Day 0, represent results obtained from at least four different cultures. * $P < 0.05$, compared with Day 0, relative to β -actin; † $P < 0.05$; ns = not significant compared with control cultures (6-day serum free media exposed to transfection reagent without siRNA). Panels E–H show phase contrast images of human ASM cells in the absence (transfection reagent alone; E) or presence of siRNA directed at integrin $\alpha 3$ (F), $\alpha 6$ (G), or $\alpha 7$ (H). Bar = 50 μ m.

regulating phenotype plasticity and heterogeneity of smooth muscle cells that may be critical biological components of fibroproliferative disorders.

Laminin is essential for the commitment and differentiation of embryonic lung mesenchyme into ASM, and for its circumferential organization around bronchi (19). In primary culture of ASM cells from adult tissues, laminin can slow modulation from the contractile phenotype to a proliferative state (4). Moreover, recent studies using bovine tracheal smooth muscle strips show that laminin, by itself, does not affect contractility or proliferation but reduced the effect of the mitogen, PDGF, on these parameters (20). This would support the notion that laminin regulates both phenotype and function of intact ASM. We recently showed that endogenously expressed laminin, likely laminin-2, is also needed for differentiated ASM cells to re-acquire a contractile phenotype after modulation to a proliferative state *in vitro* (7). This is consistent with reports showing laminin-1 and -2 to be essential for accumulation of differentiated smooth muscle during development of the airways and gastrointestinal tract (19). Though the ECM is recognized as a regulator of smooth muscle phenotype, before our study, the functional role of ECM receptor expression in mediating phenotype maturation in differentiated myocytes has not been elucidated. Notably, changes in integrin expression is associated with atherosclerosis and post-

angioplasty restenosis (21), and some studies have assessed ECM receptor expression in association with the proliferative, apoptotic, and pro-inflammatory responses of phenotypically modulated myocytes (6, 16, 22). Though there appears to be a requirement for $\beta 1$ integrins in smooth muscle growth, a precise determination of the ECM selectivity of the receptor heterodimers involved has not been reported. Thus, the current study is unique both in its focus on the role of integrins in mediating ASM cell maturation to a contractile phenotype, and on receptors with an established binding selectivity for a single ECM proteoglycan, laminin (9).

The selectivity of $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 7\beta 1$ integrin heterodimers for laminin is a determinant of α -subunit identity (9). Coordinated expression of laminin isoforms and laminin-binding integrin subunits occurs in smooth muscle of the developing vasculature and gastrointestinal tract (10), and is altered in response to tissue injury (21). However, the precise functional role of laminin-binding integrins in phenotype determination of differentiated smooth muscle cells is not known. Our experiments reveal that maturation of ASM cells is associated with increased total abundance of $\alpha 3A$ -, $\alpha 6A$ -, and $\alpha 7B$ -subunits in the absence of any changes in expression of $\beta 1$ -subunit, or $\alpha 5$ integrin, which has affinity for fibronectin. To ensure that this profile of integrin subunits is similar to that of intact tissue, we used immunoblotting, PCR, and immunohistochemistry to assess integrin expression

Figure 7. Western blot analysis showing confirmation of effect of siRNA for integrin $\alpha 3$ (A) or $\alpha 6$ (B) on protein abundance of integrin $\alpha 3A$ and $\alpha 6A$. Grouped data, which are expressed as relative units to Day 0, represent results from at least four different cultures. * $P < 0.05$, compared with Day 0, relative to β -actin; [†] $P < 0.05$; ns = not significant compared with control cultures (6 days of serum-free media exposed to transfection reagent without siRNA).

in human bronchial smooth muscle. Indeed, $\alpha 3A$, $\alpha 6A$, $\alpha 7B$, and $\beta 1$ were highly expressed. In tissue and cultured myocytes we also detected $\alpha 3B$ and $\alpha 6B$, but $\alpha 7A$ was only present in cultured cells; the abundance of each was unchanged during contractile phenotype acquisition. Interestingly, although β -subunits induce downstream signaling events that regulate cell responses such as cycle progression and actin cytoskeleton assembly (23), α -subunits and their binding to ECM ligands modulates the cascade of pathways invoked by the β -subunit, and thus the α integrins are critical in directing specific cell responses (23, 24). In this context, the selective increase in expression of total $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$ that occurs during contractile phenotype acquisition is consistent with a possible functional role for these α -integrin subunits in myocyte maturation.

TABLE 5. EFFECTS OF TREATMENT WITH $\alpha 3$ OR $\alpha 6$ INTEGRIN siRNA ON ABUNDANCE OF $\alpha 5$, AND $\alpha 7B$ PROTEIN IN 6-DAY SERUM DEPRIVED HUMAN AIRWAY SMOOTH MUSCLE CELL CULTURES

Protein Abundance	Integrin siRNA $\alpha 3$		Integrin siRNA $\alpha 6$	
	–	+	–	+
Integrin $\alpha 5$	1.0 $\pm$ 0.2*	1.9 $\pm$ 0.2 [†] *	1.1 $\pm$ 0.1*	1.9 $\pm$ 0.1 [†] *
Integrin $\alpha 7B$	1.7 $\pm$ 0.1 [†]	1.8 $\pm$ 0.1 [†] *	1.8 $\pm$ 0.2 [†]	1.6 $\pm$ 0.2 [†] *

Definition of abbreviation: siRNA, small interfering RNA.

Data are from cultures treated with transfection reagent alone (–) or integrin-selective siRNA (+). Protein abundance is expressed as fold change after Day 6 compared with Day 0 (= 1). Protein loading was corrected using β -actin abundance. Values represent mean $\pm$ SEM from duplicate experiments using four different cell cultures.

* Not significant, for comparison with abundance on Day 0, which was normalized to a value of 1.0.

[†] $P < 0.05$, for comparison with abundance on Day 0, which was normalized to a value of 1.0.

* $P < 0.05$, for comparison with Day 6 abundance in the absence of siRNA (–).

[†] Not significant, for comparison with Day 6 abundance in the absence of siRNA (–).

Heterogeneity of the cytoplasmic domains of α -subunits can result from post-translational modifications and through alternative splicing, which produces so-called A and B isoforms (9). Though the precise role of cytoplasmic domain splicing is not clear, it may underpin differences in intracellular signaling and confer unique functional roles to integrins. Indeed, as ASM responses to different laminin isoforms vary during myocyte differentiation and maturation (7, 25), changes in expression of integrin splice variants may be an associated mechanism. Our data reveal that total and cell surface integrin $\alpha 7B$, but not integrin $\alpha 7A$, was approximately doubled in abundance in ASM cell cultures induced to a contractile phenotype. This is consistent with reports indicating that integrin isoform switching is an important mechanism in skeletal muscle cell differentiation, as cytoplasmic splice variants of integrin $\alpha 7$ are differentially expressed during development (26). The presence of alternative splice variants of laminin-binding integrin $\alpha 3$ and $\alpha 6$ is also well known (9), and our data indicate the A isoform of each is selectively induced during myocyte maturation. In contrast with the B isoforms, the A cytoplasmic variants of $\alpha 3$ and $\alpha 6$ appear to be phosphorylated on serine residues, only weakly on tyrosine residues, and are a major target for PKC after phorbol ester exposure (27). No such differences in the phosphorylation states of integrin $\alpha 7$ isoforms have been documented to date; however, alternative splicing of the cytoplasmic domains for $\alpha 6$ and $\alpha 7$ is similar (26), suggesting that parallel differences in signaling profiles between $\alpha 7A$ and $\alpha 7B$ may exist.

Our experiments do not address functional differences between desmin- and integrin $\alpha 7$ -expressing human ASM subpopulations. However, in previous experiments using canine tracheal smooth muscle cells, we sorted myocytes on the basis of contractile protein content, and in subsequent subculture we observed a significantly suppressed proliferative response in cells that retained elevated abundance of smMHC and sm- α -actin (1). This suggests that the $\alpha 7B$ - and desmin-enriched human myocytes we identified may also exhibit reduced proliferative response.

Figure 8. Western blot analysis showing the effect of siRNA silencing of integrin $\alpha 3$ (A) or integrin $\alpha 6$ (B) on the accumulation of desmin and smooth muscle α -actin over 6 days of serum free culture. Grouped data, which are expressed as relative units to Day 0, represent results obtained from at least four different cultures. * $P < 0.05$, compared with Day 0, relative to β -actin; ns = not significant compared with control cultures (6 days of serum-free media exposed to transfection reagent without siRNA).

A unique aspect of this study is our use of human ASM cultures subjected to prolonged serum-free culture conditions to induce a contractile phenotype in a subset of cells, as we have reported previously (3, 14). Phenotypically heterogeneous subpopulations of smooth muscle cells in culture and intact tissues have been distinguished by differences in morphology, molecular signature, and functional properties (28–34). Consistent with published reports (3, 14), our immunofluorescence analyses using antibodies for smMHC and desmin, stringent molecular markers of contractile phenotype smooth muscle cells (11, 35), confirmed that maturation occurred in only a select subpopulation of myocytes. Thus, immunoblotting and PCR assays, which rely on sampling of all cells, do not necessarily reflect the direct association between integrin expression and myocyte maturation. To assess the association of integrin expression in those myocytes that undergo maturation, we developed novel flow cytometry-based protocols in which we prepared fluorochrome-conjugated primary antibodies for simultaneous labeling of cell surface integrin subunits and intracellular desmin. By this approach, subgroups of ASM cells comprising 20 to 40% of all myocytes were revealed with labeling for desmin or the individual laminin-binding α -integrins that increased in total abundance during phenotype maturation. Notably, myocytes that expressed $\alpha 7B$ were distinct in showing significant positive co-expression with desmin, as nearly 70% of these cells were of the contractile phenotype. Due to the sensitivity of the flow cytometry assay we developed, the exclusion cutoff used for positive integrin labeling was at the 95th percentile of control cells, thus our gating criteria were considerably more rigorous than standard single color flow cytometry where mean fluorescence intensity is compared between groups. This could explain why we did not see 100% correlation between $\alpha 7B$ and desmin co-expression, and may have reduced the apparent co-expression of $\alpha 3A$ or $\alpha 6A$ with desmin in individual cells, which was less than 50%. Nonetheless, our flow cytometry experiments are significant, as they provide first time evidence for a differential association of a single laminin-binding α -integrin splice variant, $\alpha 7B$, with contractile phenotype acquisition of human ASM cells. Moreover, these data suggest that the

pattern of laminin-binding α -integrin expression is closely related to phenotype heterogeneity of human ASM cells.

Our data from PCR, immunoblotting, immunofluorescence and flow cytometry support the existence of an intimate relationship between laminin-binding integrins and ASM cell maturation; however, they do not conclusively demonstrate a requirement for these receptors. Therefore, we developed dual-transfection siRNA protocols, which effectively abrogated the increased expression of individual target integrin proteins that would typically occur in parallel with accumulation of contractile phenotype marker proteins during prolonged serum-free culture. By this approach we demonstrated that though total $\alpha 3A$, $\alpha 6A$, and $\alpha 7B$ integrin was increased during myocyte maturation, only the accumulation of $\alpha 7B$ is required for acquisition of a contractile phenotype (Figure 5). This is consistent with a previous study by Yao and colleagues (36) showing that $\alpha 7$ expression correlates with the differentiated smooth muscle phenotype in vascular, gastrointestinal, and genitourinary systems. Moreover, as our data pertain to differentiated myocytes, they also extend recent observations from studies with an embryonic lethal $\alpha 7$ knock-out mouse, in which $\alpha 7B1$ is important for recruitment and survival of vascular smooth muscle cells during development (37). It appears that the principal effect of our $\alpha 7$ siRNA protocol is to prevent $\alpha 7B$ accumulation, as our protocols had only a marginal effect in reducing $\alpha 7A$ (not shown), which is not induced with serum withdrawal. Our siRNA strategy for $\alpha 7$ was also highly selective, as no change in accumulation of $\alpha 3$ or $\alpha 6$, or in the steady state expression of $\beta 1$ or $\alpha 5$ integrin was observed. Notably laminin-2 and -4 are exclusive ligands for integrin $\alpha 7$ (18), and in a recent study we showed that endogenously synthesized laminin-2 is required for human ASM maturation (7). Collectively our findings indicate that ASM maturation is principally manifested through the binding of endogenously expressed laminin, likely laminin-2, to integrin $\alpha 7B$, which becomes markedly increased during contractile phenotype acquisition.

The intracellular pathways necessary for $\alpha 7B$ -mediated myocyte maturation cannot be elucidated from our studies, but a number of integrin-linked pathways are also determinants of smooth

muscle phenotype. A pathway including phosphatidylinositol-3 kinase, mammalian target of rapamycin, p70S6 kinase, and 4E-BP1 is required for ASM cell hypertrophy and contractile protein accumulation in serum-free culture and in response to transforming growth factor $\beta 1$ (3, 38), insulin, or Rho-Kinase (39). Phosphatidylinositol-3 kinase signaling is implicated in integrin-mediated pathways that modulate a number of cellular processes, including differentiation and hypertrophy (40). Expression of smooth muscle specific genes, which is required for myocyte maturation, is induced via RhoA-Rho kinase signaling, and is modulated by PKC (2, 41, 42). RhoA and PKC have close ties with integrins (43), and thus they are also likely candidates for mediating $\alpha 7 \beta$ effects on myocyte maturation. Using HEK293 cell lines that express integrin $\alpha 7 \beta$, Mielenz and colleagues (44) reported that laminin binding selectively induces phosphorylation of p125^{FAK}, paxillin and p130^{CAS}; these signaling effectors are associated with regulation of cytoskeletal dynamics, which is a determinant of ASM cell contraction, migration, and the transcription of smooth muscle-specific genes such as smMHC and sm- α -actin (2, 11, 42, 45). Clearly, our present observations provide a platform for future mechanistic studies to dissect the precise signaling cascades associated with $\alpha 7 \beta$ control of smooth muscle phenotype.

We were successful in using siRNA to prevent accumulation of $\alpha 3$ - and $\alpha 6$ -integrin during prolonged serum deprivation; however, these interventions were without effect on ASM cell phenotype maturation. This was the case even with siRNA for $\alpha 6$ integrin, which serendipitously blocked induction of both $\alpha 3 \text{A}$ and $\alpha 6 \text{A}$ in serum-free culture. These data confirm that there is no requirement for increased expression of $\alpha 3$ and $\alpha 6$ in ASM contractile phenotype acquisition; however, as siRNA did not completely deplete $\alpha 3 \text{A}$ or $\alpha 6 \text{A}$ protein, it is not certain if the small amount of protein remaining may be important in supporting the effects of $\alpha 7 \beta$. This possibility seems unlikely, however, in light of our flow cytometry results that indicated fewer than half of myocytes that expressed $\alpha 3 \text{A}$ or $\alpha 6 \text{A}$ also expressed the contractile phenotype marker desmin. Interestingly, we did observe an increase in integrin $\alpha 5$ in myocytes transfected with $\alpha 3$ or $\alpha 6$ siRNA. Consistent with this finding, $\alpha 3$ -null keratinocytes display increased adhesion to fibronectin (46), which selectively binds integrin $\alpha 5 \beta 1$ (17). However, as our flow cytometry studies showed that $\alpha 5$ and $\beta 1$ integrins are expressed by all cells, future studies are required to examine whether changes in $\alpha 5$ are linked to those cells that undergo phenotype maturation.

In summary, we show that laminin-2 (7) and laminin-binding integrin expression is concomitantly induced in ASM cells under conditions that promote acquisition of a contractile phenotype. Moreover, we show that $\alpha 7 \beta$ integrin is unique, as it is required for myocyte maturation to occur. These data reveal new biological mechanisms relevant to fibroproliferative disorders such as asthma and atherosclerosis that are characterized by the accumulation of smooth muscle, and in which smooth muscle phenotype switching occurs (47, 48). At present, though differences in ECM composition are reported in the airways of patients with asthma, it is not known if changes in integrin expression, in particular on ASM cells, are directly associated with disease progression (49). Notably, in patients with congenital muscular dystrophy due to laminin $\alpha 2$ chain deficiency, and in *dy/dy* mice that do not express laminin $\alpha 2$ chain, $\alpha 7$ integrin expression becomes severely diminished, indicating that changes in integrin abundance can be directly affected by changes in the ECM (50). Furthermore, as changes in $\alpha 7$ integrin expression are associated with the pathogenesis of vascular remodeling in response to injury (21), future studies are clearly necessary to assess the functional role of $\alpha 7$ integrin expression in other disease states, such as asthma. Though $\alpha 7$ integrin knockout mice have been

generated (37), the mutation is embryonic lethal, thus more sophisticated transgenic mouse models that target this gene are necessary to investigate its role in disease models.

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