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Skeletal myosin heavy chain function in cultured lung myofibroblasts

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Myofibroblasts are unique contractile cells with both muscle and nonmuscle properties. Typically myofibroblasts are identified by the expression of α smooth muscle actin (ASMA); however some myofibroblasts also express sarcomeric proteins. In this study, we show that pulmonary myofibroblasts express three of the eight known sarcomeric myosin heavy chains (MyHCs) (IIa, IId, and embryonic) and that skeletal muscle myosin enzymatic activity is required for pulmonary myofibroblast contractility. Furthermore, inhibition of skeletal myosin activity and myofibroblast contraction results in a decrease in both ASMA and skeletal MyHC promoter activity and ASMA protein expression, suggesting a potential coupling of skeletal myosin activity and ASMA expression in myofibroblast differentiation. To understand the molecular mechanisms whereby skeletal muscle genes are regulated in myofibroblasts, we have found that members of the myogenic regulatory factor family of transcription factors and Ca^{2+}-regulated pathways are involved in skeletal MyHC promoter activity. Interestingly, the regulation of skeletal myosin expression in myofibroblasts is distinct from that observed in muscle cells and suggests that cell context is important in its control.

Introduction

Myofibroblasts are unique cells, possessing ultrastructural characteristics of both muscle and nonmuscle cells. They are essential for the formation of functional adult tissues and are intimately involved in tissue homeostasis (for reviews see Desmouliere and Gabbiani, 1996; Powell et al., 1999; Walker et al., 2001). However, the persistence of these cells after injury and their continued secretion of ECM have implicated them in various fibroproliferative processes, including fibrotic diseases of the liver, kidney, and lung (Hautekeete and Geerts, 1997; Powell et al., 1999; Badid et al., 2001; Phan 2002). In vivo, myofibroblasts are thought to arise from the transient differentiation of resident fibroblasts through multiple paracrine-mediated pathways including TGFβ mechanisms (for reviews see Desmouliere and Gabbiani, 1996; Vaughan et al., 2000). The expression of α smooth muscle actin (ASMA) and various intermediate filaments, e.g., desmin and vimentin, is most frequently used to identify myofibroblasts. Other characteristic properties include the secretion of ECM and contractility (for reviews see Serini and Gabbiani, 1999; Tomasek et al., 2002). Indeed, ASMA has been implicated in the contractility of dermal, gingival, periodontal ligament, and pulmonary myofibroblasts (Arora and McCulloch, 1994; Germain et al., 1994; Hinz et al., 2001), and inhibition of ASMA expression blocks chicken embryonic myofibroblast contraction (Feugate et al., 2002). Myofibroblasts have the unusual property of expressing not only smooth muscle proteins, but also a number of sarcomeric proteins otherwise found only in skeletal muscle, including several skeletal-specific myosin heavy chain (MyHC) isoforms (Mayer and Leinwand, 1997; van der Ven and Furst, 1998).

Myosin is the ATP-dependent motor protein that, together with actin, is responsible for sustaining contraction in muscle (Huxley, 1969). There are eight isoforms of sarcomeric MyHC, six of which are expressed in skeletal muscle (Weiss and Leinwand, 1996). Different MyHC expression profiles are observed in myofibroblasts depending upon their tissue of origin. For example, cultured kidney myofibroblasts express six MyHC isoforms, whereas liver myofibroblasts express a

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Key words: myofibroblast; skeletal myosin; actin; contraction; lung

Abbreviations used in this paper: ASMA, α smooth muscle actin; BDM, 2,3-butanedione monoxime; BTS, *N*-benzyl-*p*-toluene sulfonamide; ca, constitutively active; CaM, calmodulin; CMV, cytomegalovirus; cn, constitutively nuclear; CsA, cyclosporin A; MEF2, myocyte enhancer factor 2; MRF, myogenic regulatory factor; MyHC, myosin heavy chain; NFAT, nuclear factor of activated T cells; VIC, valvular interstitial cell.

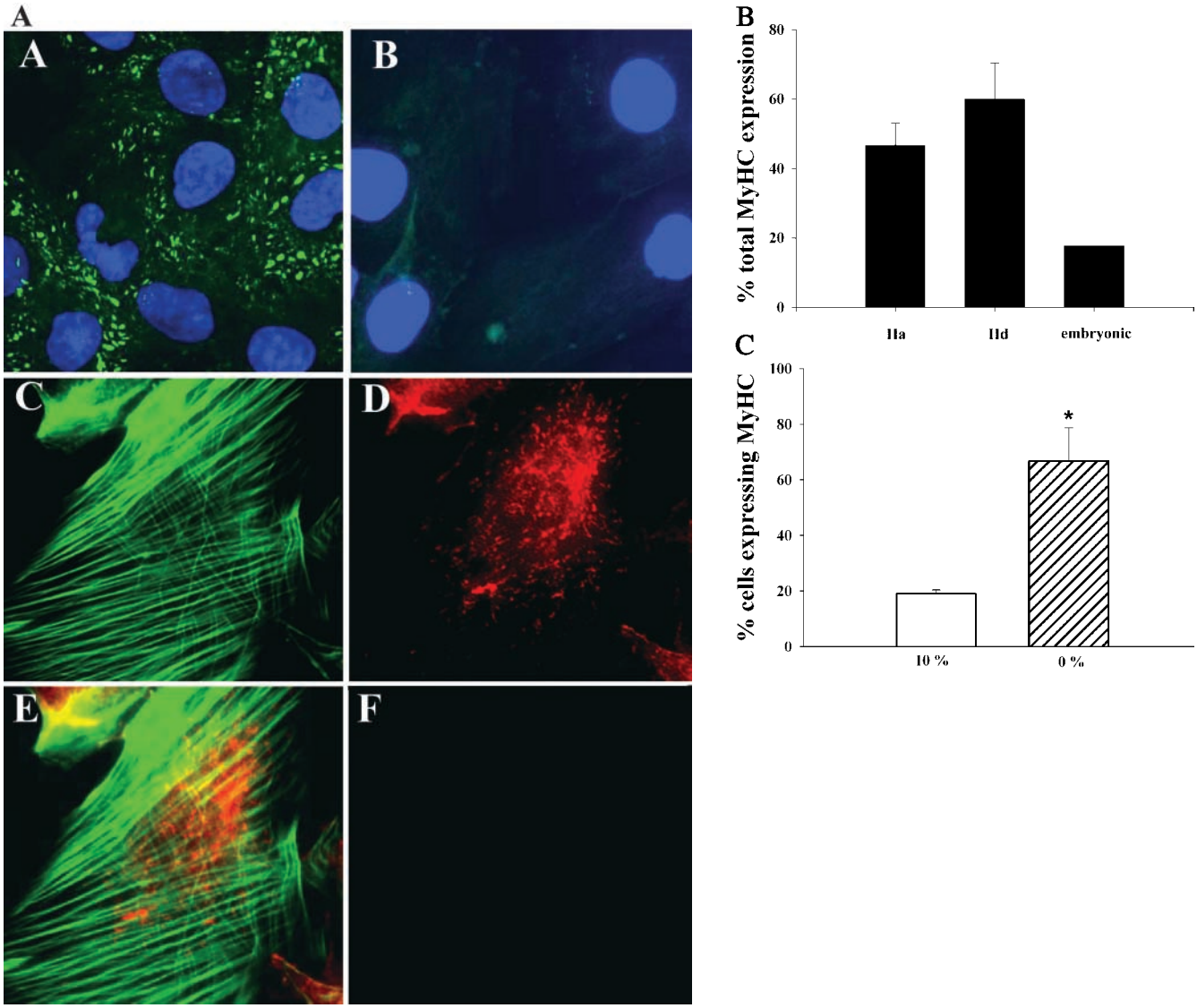

Figure 1. **Quantification of MyHC and ASMA expression in pulmonary myofibroblasts by immunostaining and RT-PCR.** (A) Cells were grown in 10% FBS, transferred to serum-free media for 48 h, and stained as described in the Materials and methods. (panel A) Skeletal MyHC, all isoforms. (panel B) Secondary Ab alone. (panel C) ASMA. (panel D) MyHC IId. (panel E) Merged panels C and D. (panel F) Secondary Ab alone. (B) Pulmonary myofibroblasts express IIa, IId, and embryonic skeletal MyHC. RT-PCR analysis was performed as described in the Materials and methods. (C) Skeletal MyHC is expressed at higher levels under quiescent conditions. Cells were grown in either 10% FBS or 0% FBS and stained for MyHC expression as described in the Materials and methods. Values represent the number of positive MyHC cells/total number of cells. Experiments were performed a minimum of three times with 100–200 total cells per experiment. Error bars represent SEM, with * indicating $P < 0.05$.

subset of those expressed in kidney myofibroblasts (Mayer and Leinwand, 1997). Although the role of sarcomeric MyHCs in myofibroblasts is not understood, it is logical to hypothesize that they participate in, or are required for, myofibroblast contractility, an important aspect of their function in vivo.

What factors regulate skeletal muscle gene expression, specifically MyHC expression, in these cells is also unclear. Active myogenic regulatory factors (MRFs), a family of skeletal muscle–specific transcription factors, are expressed in a number of myofibroblasts (Rockey et al., 1993; Mayer and Leinwand, 1997; Kaminski et al., 2000). MRFs can activate the entire skeletal muscle gene program when expressed exogenously in a number of cell types (Weintraub et al., 1989). Although no evidence exists for direct regulation of skeletal MyHC genes by MRFs, MyoD indirectly regulates the transcriptional activity of the skeletal MyHC IIb gene (Takeda et al., 1995; Lakich et al., 1998; Wheeler et al., 1999). The calcineurin and calmodulin (CaM) kinase pathways have been shown to differentially regulate MyHC isoform expression in muscle cells (Allen et al., 2001; Allen and Leinwand, 2002). Calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factors, allowing their nuclear transport (Chin et al., 1998). CaM kinase can regulate transcription by phosphorylation of myocyte enhancer factor 2 (MEF2), resulting in nuclear export of the repressor protein histone deacetylase (Lu et al., 2000; McKinsey et al., 2000). Whether these pathways reg-

ulate MyHC expression in myofibroblasts is addressed in the current study.

This work focuses on delineating the expression pattern, mechanism of regulation, and function of skeletal MyHC within the context of the pulmonary myofibroblast. Pulmonary myofibroblasts are implicated in various pulmonary interstitial diseases. As a result, much is known about the signaling pathways that are responsible for myofibroblast activation in lung, including extensive data on TGFβ-induced myofibroblast proliferation and regulation of ASMA expression at both the cellular and molecular levels (Phan et al., 1999; Pardo and Selman, 2002; Phan, 2002). An understanding of those mechanisms responsible for the contractile function of pulmonary myofibroblasts, however, has lagged considerably. Our findings reported herein show that pulmonary myofibroblasts express three of the eight sarcomeric MyHC isoforms, and their expression is differentially regulated with respect to each other and in comparison with skeletal muscle (Allen et al., 2001). Furthermore, we demonstrate that while sarcomeric myosin is not necessary for the proliferation of these cells, its enzymatic activity is essential for their contraction. This is the first report to link skeletal MyHC with the contraction of myofibroblasts.

Results

As both kidney and liver myofibroblasts express several skeletal MyHC isoforms (Mayer and Leinwand, 1997), we wished to examine the potential role of skeletal MyHC in pulmonary myofibroblast function. Specifically, (1) Do pulmonary myofibroblasts express skeletal MyHC(s); (2) Is skeletal MyHC activity required for myofibroblast contractility; (3) What is the relationship between skeletal MyHC and ASMA with respect to contractility; and (4) What signaling pathways regulate MyHC expression?

Pulmonary myofibroblasts express sarcomeric myosin

Primary rat pulmonary myofibroblasts do express skeletal MyHC, and it is localized in a punctate and perinuclear pattern (Fig. 1 A). Furthermore, skeletal MyHC does not appear to colocalize with ASMA in stress fibers (Fig. 1 A). Of the six skeletal MyHC isoforms, pulmonary myofibroblasts express the IIa, IId, and embryonic isoforms, with IIa and IId being the most abundant (Fig. 1 B). Perinatal and IIb isoforms were undetectable (not depicted).

The differentiated myofibroblast phenotype, characterized in part by the expression of ASMA, is very plastic under different growth conditions (Arora and McCulloch, 1999). Therefore, to investigate whether altered growth conditions could change the MyHC gene expression profile of myofibroblasts, the effect of serum on the presence of MyHC was evaluated by immunostaining. Under normal proliferating conditions, myosin was expressed in ~20% of the myofibroblasts (Fig. 1 C); however, removal of serum from the media resulted in a greater than threefold increase in the number of cells expressing sarcomeric myosin. The increase in skeletal MyHC expression under serum-free, nonproliferating conditions, i.e., quiescent, is consistent with that observed for ASMA expression in gingival myofibroblasts. Under quiescent conditions in these cells, ASMA turnover is slow, more ASMA is incorporated into stress fibers, and cells are resistant to apoptosis. In contrast, gingival myofibroblasts growing in serum turn over ASMA protein and mRNA quickly and are not as phenotypically stable (Arora and McCulloch, 1999).

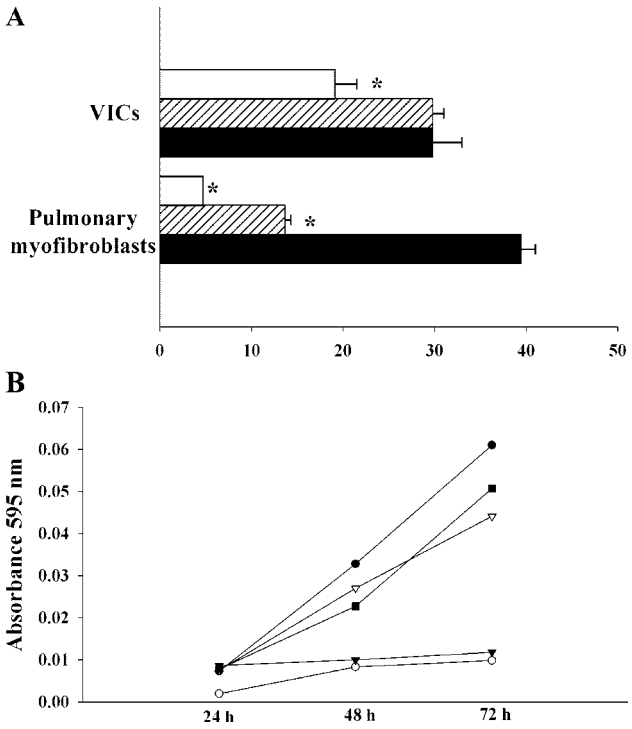

Figure 2. **Inhibition of skeletal MyHC prevents myofibroblast-mediated collagen matrix contraction but not myofibroblast proliferation.** (A) Collagen matrices were prepared as described in the Materials and methods. The percent contraction represents the diameter of the matrix after contraction normalized to the diameter before matrix release under normal conditions or in the presence of inhibitors: white, 15 mM BDM; hatched, 10 μM BTS; black, no inhibitor. Error bars represent SEM, with * representing $P < 0.05$. (B) Myofibroblast proliferation was assayed as described in the Materials and methods under normal growth conditions or in the presence of various inhibitors of myosin as follows: closed circle, 10% serum; open circle, 1% serum; closed triangle, 15 mM BDM; open triangle, 5 μM BTS; closed square, 10 μM BTS; $n = 3$.

Sarcomeric MyHC is necessary for pulmonary myofibroblast contraction but not proliferation

The most logical role for skeletal MyHC isoforms would be in myofibroblast contractility. To test this hypothesis, collagen matrix contraction assays were performed in the presence and absence of a small molecule inhibitor of skeletal myosin II activity, *N*-benzyl-*p*-toluene sulfonamide (BTS). Under normal growth conditions (10% FBS), pulmonary myofibroblasts contract collagen matrices by ~40% of their original diameter (Fig. 2 A). Others have observed similar basal levels of pulmonary myofibroblast contraction (Hinz et al., 2002). When myofibroblasts were treated with BTS, matrix contraction was significantly inhibited to <14% of the original diameter, similar to results obtained when cells were treated with the more promiscuous myosin inhibitor 2,3-butanedione monoxime (BDM) (Fig. 2 A). Unlike BDM, which inhibits all type II myosin isoforms, BTS is

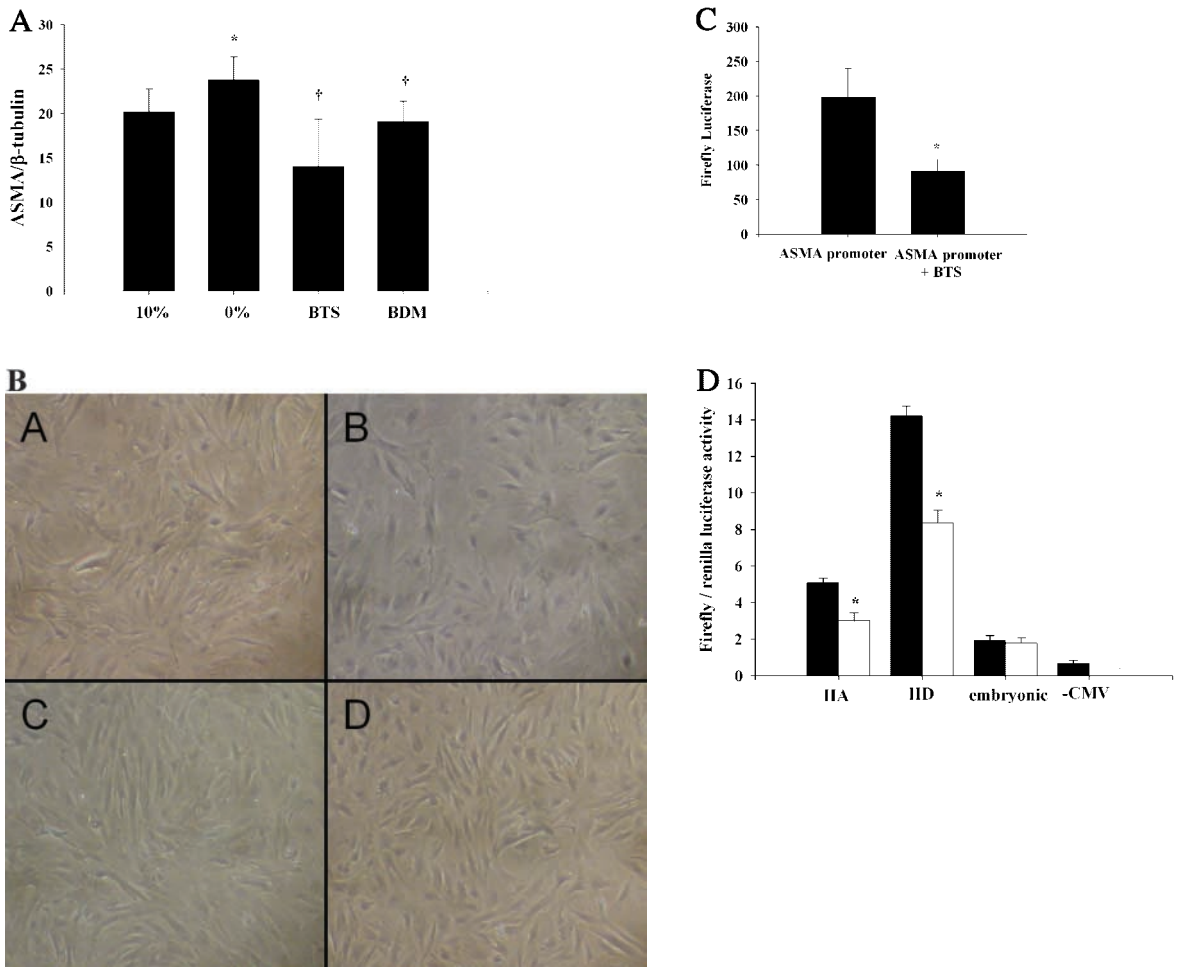

Figure 3. **Inhibition of skeletal MyHC activity down-regulates ASMA expression.** (A) Myofibroblasts were grown in 10% FBS, transferred to 0% FBS, 0% FBS + 10 μM BTS, or 0% FBS + 15 mM BDM for 48 h, and probed for ASMA expression as described in the Materials and methods. Changes in ASMA expression are normalized to endogenous β-tubulin expression. * indicates significance between 10% and 0% ($P < 0.001$); † indicates significance between 0% and treated cells ($P < 0.05$). (B) Light microscopy of myofibroblasts grown for 48 h in (panel A) 10% serum, (panel B) 0% serum, (panel C) 15 mM BDM, and (panel D) 5 μM BTS. (C) Activity of the rat ASMA promoter in pulmonary myofibroblasts in the presence or absence of 10 μM BTS. Data are normalized to a promoterless vector control (−CMVluc), as promoter interference is observed upon cotransfection with a *Renilla* luciferase construct. Results are given as means ± SEM; $n = 3$. * indicates a significant difference ($P < 0.001$). (D) Activity of MyHC promoters in the presence of BTS. Approximately 1.0 kb of promoter region for each MyHC isoform was transfected into myofibroblasts grown in 1% serum (black) or 1% serum + 10 μM BTS (white), as described in the Materials and methods. A promoterless vector (−CMVluc) served as a negative control. Results are given as means ± SEM from three experiments. * indicates a significant difference ($P < 0.01$).

specific for skeletal type II myosin (Cheung et al., 2002). While the specificity of BTS on purified myosin has been established (Cheung et al., 2002), as an additional control, we tested the ability of BTS to inhibit the contraction of valvular interstitial cells (VICs). VICs are heart valve myofibroblast cells (Messier et al., 1994, 1999) that do not express any skeletal MyHC isoforms (Walker, G., personal communication). When collagen matrices containing VICs are treated with BTS, matrix contraction is not blocked and occurs as well as that observed under normal growth conditions (Fig. 2 A). BDM treatment, however, significantly inhibits VIC contraction by ∼25%, as expected. Because myosin expression is greatest in pulmonary myofibroblasts under low or serum-free conditions, it would be ideal to measure contractility in these conditions. However, this was experimentally not possible, because after trypsinization, the cells did not obtain the proper morphology inside the collagen matrices. When the two myosin inhibitors were tested for effects on proliferation, only BDM blocked cell proliferation to the same extent as removal of serum (Fig. 2 B). BTS at either 5 or 10 μM ($IC_{50} = 5$ μM) had no significant effect on cell proliferation. Such results indicate that skeletal-specific MyHC activity is required for myofibroblast contractility but not proliferation.

Skeletal MyHC activity is linked to an increase in ASMA expression

A key phenotypic feature of myofibroblast differentiation is an increase in ASMA expression (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993; Serini and Gabbiani, 1999). As a decrease in ASMA expression has been shown to reduce both lung fibroblast contraction and granulation tissue wound closure (Feugate et al., 2002; Hinz et al., 2002), we hypothesized that MyHC expression and activity would

correlate with ASMA expression. Indeed, when cell lysates were examined for changes in ASMA expression, a significant increase in ASMA expression was observed when myosin expression was highest (Fig. 3 A). Furthermore, inhibition of MyHC activity with either BTS or BDM resulted in a concomitant reduction in ASMA expression (Fig. 3 A). The reduction is due to a specific decrease in the concentration of ASMA/cell and is not due to a decrease in the total number of cells expressing ASMA (unpublished data). Furthermore, the decline in ASMA expression is not due to induced cytotoxicity, as the treated cells are morphologically similar to those grown under normal conditions (Fig. 3 B). The ASMA promoter has recently been shown to be mechanotranscriptionally regulated through the application of tensile forces through a force-responsive CArG-box element (Wang et al., 2002). Therefore, we examined the effect of inhibiting MyHC activity, and therefore contractility, on ASMA promoter activity. When pulmonary myofibroblasts were transfected with a reporter gene construct downstream of the rat ASMA promoter and treated with 10 μM BTS, a greater than twofold reduction in ASMA promoter activity was observed (Fig. 3 C). These results are consistent with previous reports showing that upon force application, ASMA promoter activity is increased in osteoblasts by ~60% (Wang et al., 2002). Such mechanical regulation of ASMA suggests that other contractile genes in myofibroblasts, in particular MyHC, may also be regulated to some degree through mechanosensory regulation.

The proximal ~1-kb promoter regions of the three adult fast MyHC isoforms have previously been characterized in muscle cells and have been shown to confer muscle-specific and differentiation-sensitive expression in vitro (Allen et al., 2001). Additionally, the ~1-kb sequence of the embryonic promoter used for this study has also been determined to drive differentiation-specific activity (Belykin, D., personal communication). Indeed, when the activities of the upstream promoter regions of the three MyHCs expressed in these cells (IIa, IId, and embryonic) were assayed in the presence of BTS, significant decreases in the activities of the IIa and IId promoters were observed (Fig. 3 D). No significant decrease in embryonic MyHC promoter activity was observed, consistent with BTS inhibition being specific for adult fast type II myosin isoforms (Cheung et al., 2002). Taken together, such results suggest that myofibroblast differentiation, in addition to growth factor regulation, may be controlled by the application of mechanical forces that in turn lead to the expression of both MyHC and ASMA, increased myofibroblast contraction, and a more differentiated phenotype.

Pulmonary myofibroblasts express muscle-specific transcriptional regulators

As sarcomeric MyHC expression appears to be an integral part of myofibroblast differentiation and function, and yet these are not muscle cells, it is of interest to determine what molecular pathways are operating in myofibroblasts to regulate muscle-specific genes. Regulators from two major families of transcription factors play a key role in skeletal muscle gene expression. The MyoD family of MRFs consists of MyoD, myf-5, myogenin, and MRF4 (for review see Molkentin and Olson, 1996). MRFs function by heterodimerizing with E-proteins and subsequently activate transcription by binding to E-box sequences (CANNTG) found in many muscle-specific genes (Lassar et al., 1991). Although MRFs are largely restricted to cells of muscle lineage, they can be expressed in various myofibroblasts, including retinal pericytes (Rockey et al., 1993), cardiac valve cells (Walker, G., personal communication), liver stellate cells (Mayer and Leinwand, 1997), and kidney myofibroblasts (Mayer and Leinwand, 1997). One MRF, myf-5, was expressed in all pulmonary myofibroblasts (Fig. 4 A); myf-5 expression was also independently verified at the RNA level by RT-PCR (not depicted). Neither myogenin nor MyoD were detectable in these cells (not depicted). Furthermore, myf-5 is also expressed in vivo in the pulmonary alveoli at one day of postnatal life, consistent with previous reports demonstrating the requirement of myofibroblasts in alveolar development and septation (Fig. 4 A) (Bostrom et al., 1996). To assess whether myf-5 is not only present but also active in these cells, a "sensor" construct containing three tandem consensus E-box binding sites linked to chloroamphenicol transferase (CAT) (Wentworth et al., 1991) was transfected into pulmonary myofibroblasts and assayed for activity. As shown in Fig. 4 B, there was significant CAT activity in pulmonary myofibroblasts transfected with the reporter construct. This construct is not active in nonmuscle mouse L-cells (Mayer and Leinwand, 1997). A construct in which the E-box sites have been mutated was inactive in myofibroblasts. Such results suggest that endogenous myf-5 in myofibroblasts is capable of binding to DNA and activating transcription of downstream genes.

The second family of transcriptional activators intimately involved in muscle determination is the MEF2 group of MADS box regulators (for review see Black and Olson, 1998). MEF2 proteins bind to AT-rich elements located in muscle regulatory sequences. Using a similar sensor strategy as above, MEF2 activity was observed in pulmonary myofibroblasts (Fig. 4 C). There was no reporter activity with a construct lacking the AT-rich MEF2 binding sites. Taken together, our results suggest that those mechanisms otherwise found primarily in skeletal muscle are present in myofibroblasts, likely resulting in transcriptional activation and regulation of muscle genes.

Regulation of skeletal muscle MyHC promoter activity in pulmonary myofibroblasts

To investigate the cis and trans elements involved in regulating MyHC expression in myofibroblasts, we compared the activities of the upstream promoter regions of the three MyHCs expressed in these cells: IIa, IId, and embryonic. In addition, we tested the activity of the adult fast IIb MyHC promoter that is not expressed in these cells. The promoters of the IIa, IId, and embryonic MyHC genes were active in pulmonary myofibroblasts and demonstrated relative activities that mirrored the endogenous levels of each isoform, i.e. IId > IIa > embryonic (Fig. 5 A). Activity of the IIb promoter was barely detectable. Furthermore, the activities of the two adult fast promoters, IIa and IId, increased when cells were grown in low serum media (Fig. 5 A), suggesting that the observed increase in myosin protein expression un-

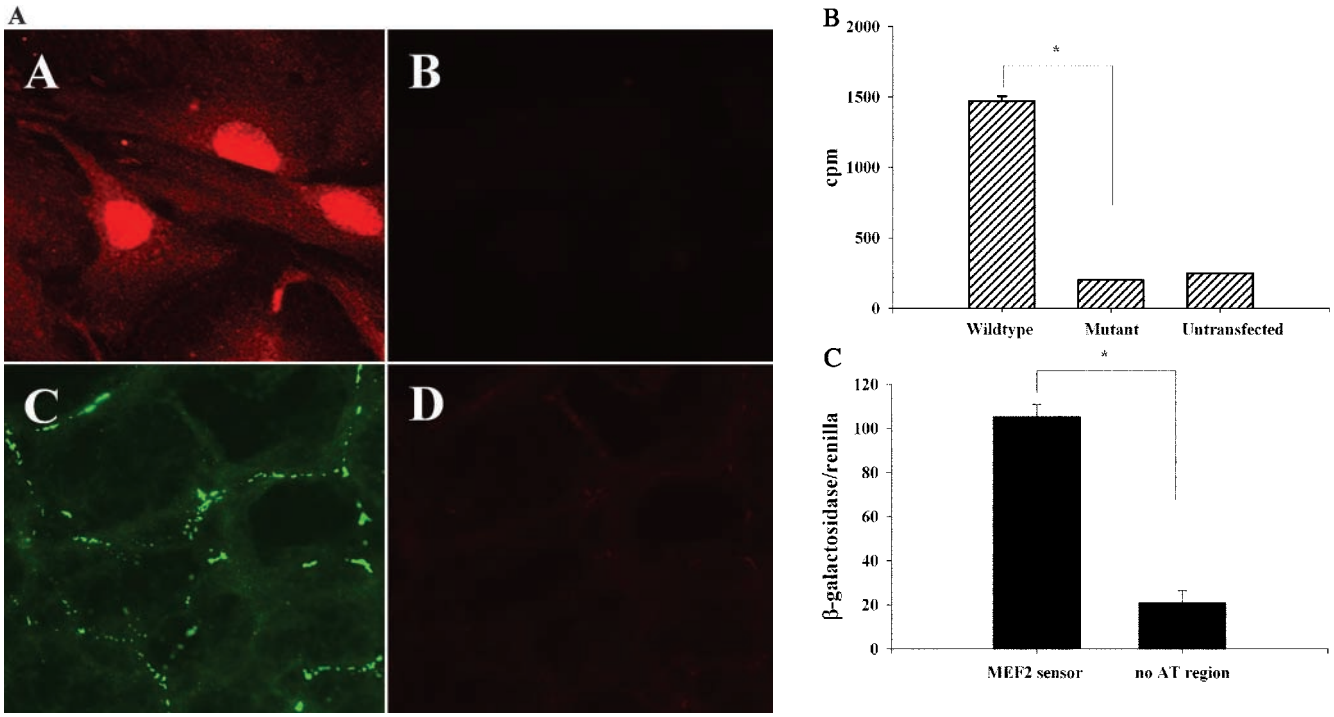

Figure 4. **Active MRFs are expressed in pulmonary myofibroblasts in vitro and in vivo.** (A) Myofibroblasts were grown in 10% FBS and transferred to 0% FBS media for 48 h. Myf-5 expression was visualized using an anti–myf-5 primary Ab and a Texas red–conjugated secondary Ab (panel A). Secondary Ab alone (panel B). Myf-5 staining in vivo in lung sections from 1-dpn mice (panel C). (B) E-box–dependent expression of muscle reporter constructs in pulmonary myofibroblasts. Myosin light chain reporter constructs (Wentworth et al., 1991) were transfected into myofibroblasts. The wild-type construct contains three E-box motifs that are mutated in the mutant construct. CAT activity was determined using ^{14}C-labeled chloroamphenicol as substrate. A representative dataset for three separate experiments is given. * denotes $P < 0.05$. (C) MEF2-sensitive expression of β-galactosidase in myofibroblasts. Reporter constructs containing either three MEF2 binding sites proximal to β-galactosidase or no MEF2 binding sites were assayed in pulmonary myofibroblasts. MEF2-dependent β-galactosidase activity is normalized to an internal cotransfected *Renilla* luciferase plasmid.

der quiescent conditions may be accomplished by transcriptional regulation. The embryonic promoter showed no significant change with modification of serum levels.

Given that members of both the MRF and MEF families of transcription factors are active in pulmonary myofibroblasts, and three different MyHC isoforms are simultaneously expressed, we sought to identify any differential responsiveness the three different promoters may have for individual transcription factors or signaling molecules. Previously, it was shown in skeletal muscle C2C12 cells that while all three adult fast promoters (IIa, IIb, and IId) possess multiple E-boxes, only the IIb promoter is responsive to MRF overexpression (Allen et al., 2001). This is not the case in the context of the myofibroblast; both the IId and embryonic promoters were significantly increased in response to MyoD overexpression, with the embryonic promoter yielding the greater response, 171 vs. 70%, respectively (Fig. 5 B). No significant increase in IIa promoter activity was observed. Overexpression of myf-5 did not result in any enhancement in promoter activity for any of the three MyHC promoters tested in this study (unpublished data). Such results suggest that different MRFs likely play distinct roles in MyHC gene regulation. Furthermore, it may be that saturating levels of myf-5 protein already exist in myofibroblasts, and higher concentrations are not required for full activity of the MyHC promoters.

Initiation of various signaling pathways via fluctuations in intracellular Ca^{2+} levels is a primary regulator of muscle contraction. More specifically, increases in intracellular Ca^{2+} activate the three adult MyHC promoters in muscle cells, with an almost 15-fold effect on the IIa promoter (Allen and Leinwand, 2002). Although changes in Ca^{2+} influx have not been directly assessed in pulmonary myofibroblast differentiation, activation of liver myofibroblasts is correlated with an up-regulation of L-type voltage-operated Ca^{2+} channels, increase in Ca^{2+} influx, and cell contraction (Bataller et al., 2001). Therefore, we investigated the role of the Ca^{2+}-regulated calcineurin/NFAT signaling pathway on MyHC promoter activity in myofibroblasts. When constitutively active (ca) calcineurin was overexpressed in pulmonary myofibroblasts, there was a resultant increase in the activity of all three MyHC promoters (Fig. 5 B). IId promoter activity was increased by ~250% over basal levels, IIa by ~200% and embryonic by ~90%. Likewise, when the downstream effector of calcineurin, NFAT, was overexpressed, a similar pattern of promoter activity enhancement was observed (Fig. 5 B), although the increase in IId activity was not as great as with ca-calcineurin. Interestingly, in muscle cells, overexpression of constitutively nuclear (cn) NFAT also preferentially increased IIa promoter activity but decreased IId activity (Allen et al., 2001).

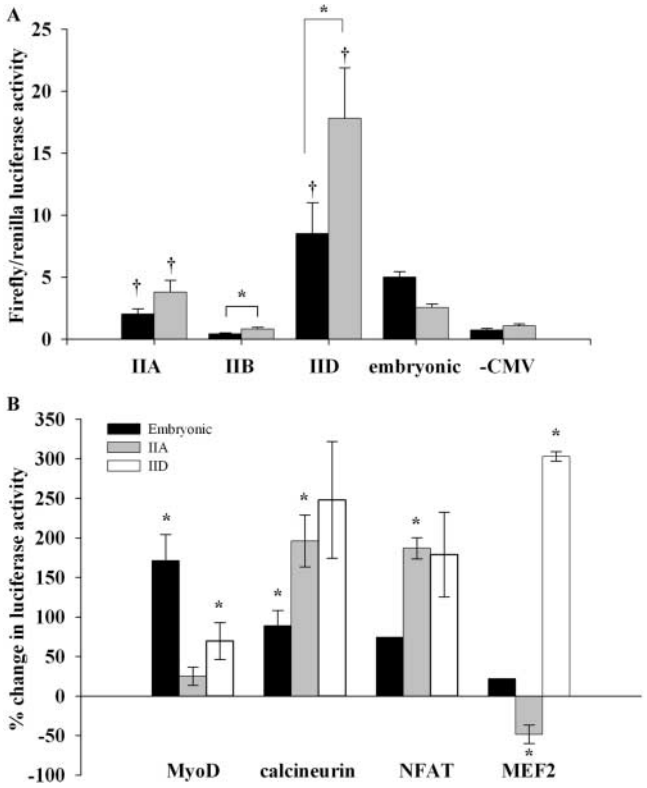

Figure 5. **Promoter activity of the MyHC IIa, IIb, IId, and embryonic promoters.** (A) Promoter regions for each MyHC isoform were transfected into myofibroblasts grown in either 10% (black) or 1% (gray) serum as described in the Materials and methods. A promoterless vector (−CMVluc) served as a negative control. Results are expressed as means ± SEM; $n = 4–5$. * indicates a significant difference ($P < 0.05$) between cells grown in 10% versus 0% serum; † indicates significance ($P < 0.01$) between IIa and IId promoter activity grown under the same conditions. (B) Differential responsiveness of the MyHC promoters to transcription factor overexpression in myofibroblasts. Myofibroblasts were cotransfected with either the embryonic (solid), IIa (gray), or IId (white) MyHC promoters and with plasmids overexpressing cn-NFAT, ca-calcineurin, MEF2c, or MyoD. Data are reported as means ± SEM of the percent increase over basal promoter activity alone; $n = 4–5$. * indicates a significant increase ($P < 0.05$) over basal promoter activity.

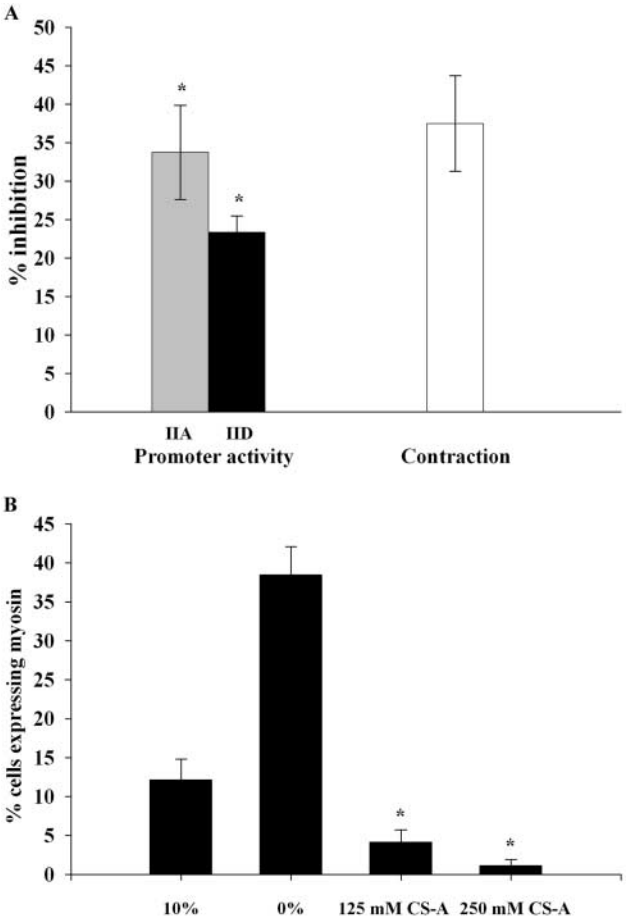

Figure 6. **Effects of CsA on myofibroblast phenotypes**. (A) IIa (gray) and IId (black) MyHC promoter activity and myofibroblast contraction (white) were measured after treatment for 36 h with 250 mM CsA as described in the Materials and methods. Results are given as the percent inhibition from either basal promoter activity or contraction. * indicates significance ($P < 0.05$). (B) Myofibroblasts were grown for 48 h in either 10% serum, 0% serum, or in 0% + CsA at the indicated concentrations. Endogenous MyHC expression was visualized using an antiskeletal muscle MyHC primary Ab and FITC-conjugated secondary Ab. Values represent the number of positive MyHC cells/total number of cells. Experiments were performed on a minimum of 200–400 total cells. Error bars represent SEM. * denotes significance ($P < 0.001$).

As MEF2 is active in pulmonary myofibroblasts, we tested the hypothesis that MEF2 is involved in the differential expression of the adult fast MyHC promoters, i.e., that MyHC IId is more highly expressed than MyHC IIa. Consistent with that hypothesis, overexpression of MEF2c significantly enhanced the activity of IId (300%), while it significantly suppressed IIa promoter activity 50% below basal levels (Fig. 5 B). These results are intriguing given that both adult fast MyHC proximal promoters share a 100% conserved AT-rich element that binds MEF2c in muscle cells (Allen et al., 2001). No significant effect was observed with the embryonic promoter, which does not contain a MEF2 binding site.

Inhibition of calcineurin blocks MyHC expression and function

In contrast to skeletal muscle cells (Allen et al., 2001), calcineurin and NFAT appear to activate both IIa and IId promoters to the same extent in pulmonary myofibroblasts. Therefore, to understand further how the calcineurin pathway affects not only MyHC expression but also myofibroblast function, we treated cells with the calcineurin inhibitor cyclosporin A (CsA) and assayed various cellular outputs, including myosin promoter activity, protein expression, and contractility. When pulmonary myofibroblasts were treated with 250 mM CsA after transfection with either the IIa or IId MyHC promoter, significant inhibition of basal promoter activities, ∼35 and ∼25%, respectively, resulted (Fig. 6 A). Furthermore, treatment of cells with CsA significantly decreased the amount of endogenous myosin expressed (Fig. 6 B) and also inhibited myofibroblasts from contracting by ∼35% (Fig. 6 A). Myofibroblasts grown in the presence of CsA (either 125 or 250 mM) had greater than eightfold reduction in skeletal myosin expression compared with cells

grown in media with no serum. These results indicate a relationship between calcineurin activation and myofibroblast contraction via MyHC expression and further strengthen the argument that MyHC expression and activity are essential components of the differentiated myofibroblast phenotype.

Discussion

Although first described in 1971 by Gabbiani et al., myofibroblasts remain one of the most enigmatic of cell types. It is now generally accepted that the modulation of these mesenchymal cells from fibroblasts to myofibroblasts is essential in tissue remodeling and normal wound healing of multiple organs through ECM and tensile force generation (Desmouliere and Gabbiani, 1996; Powell et al., 1999; Serini and Gabbiani, 1999). In this article, we have focused on understanding the molecular mechanisms of pulmonary myofibroblast differentiation and function in light of their importance in various interstitial diseases of the lung (Phan et al., 1999; Phan, 2002).

One of the best-characterized markers of the myofibroblast phenotype is ASMA. ASMA is expressed during myofibroblast differentiation, and its incorporation into stress fibers is correlated with increases in the contractile activity of fibroblastic cells in vitro (Arora and McCulloch, 1994; Hinz et al., 2001). Conversely, an understanding of skeletal muscle protein expression and regulation in these cells has lagged significantly behind that of this smooth muscle gene. To date, no one has investigated the expression of skeletal muscle proteins in pulmonary myofibroblasts or skeletal myosin function in any myofibroblasts. In this work, we demonstrate that pulmonary myofibroblasts express three of the six skeletal isoforms of MyHC, including two adult isoforms, IIa and IId, and the developmental embryonic isoform. The simultaneous expression of developmental and adult myosins suggests that the normal temporal regulation of the myosin gene cluster present in muscle is somehow interrupted in myofibroblasts. Such coexpression of adult and developmental isoforms has been seen in liver and kidney myofibroblasts as well (Mayer and Leinwand, 1997).

We also found that the number of cells expressing skeletal myosin is significantly increased under no or low serum conditions, conditions that represent a more differentiated myofibroblast (Arora and McCulloch, 1999). Consistent with increases in MyHC protein expression under less proliferative conditions, MyHC is not involved in the growth of myofibroblasts, as their proliferation in the presence of the skeletal myosin inhibitor BTS is equivalent to that seen under normal growth conditions. These data suggest a correlation between myosin expression and the modulation of myofibroblasts from a proliferative to a more quiescent phenotype. Tomasek et al. (2002) have proposed a twofold model for the transition of fibroblasts to myofibroblasts whereby "protomyofibroblasts," characterized by the absence of ASMA (cytosolic actins are present), represent the initial step in differentiation, and fully differentiated myofibroblasts, distinguished by the de novo expression of ASMA, arise from protomyofibroblasts. Based upon on the results in this work, we propose that skeletal myosin is a specific marker for identifying the differentiated myofibroblast phenotype.

An essential function of myofibroblasts is their ability to generate tensile forces in wound healing and pathological contracture (for review see Tomasek et al., 2002). Although the capacity of myofibroblasts to elicit and sustain isotonic contractile force is correlated with increases in ASMA expression (Vaughan et al., 2000; Hinz et al., 2001), the cellular mechanism by which force generation is increased is not clearly established. There is much evidence supporting the necessity for stress fiber formation in myofibroblast contraction (Burridge, 1981; Tomasek et al., 1992; Katoh et al., 1998), and the incorporation of ASMA into these fibers seems important for force production as well (Arora and McCulloch, 1994; Hinz et al., 2001, 2002). In this paper, we have identified skeletal MyHC activity as a key regulator of pulmonary myofibroblast contraction by showing that inhibition of skeletal myosin activity significantly reduced contractility. It should be noted that based upon the experimental design of the present work, the role of MyHC in tractional (migration) versus contractional forces cannot be discriminated. However, the involvement of skeletal isoforms of MyHC in developing any type of tension in nonmyogenic cells is significant. A recent report has demonstrated that transfection of ASMA into fibroblasts induces contraction in the absence of smooth muscle MyHC with no correlating increase in nonmuscle MyHC expression (Hinz et al., 2001). However, that study did not examine the expression of skeletal MyHC. The fact that myofibroblasts from different tissues express distinct sets of MyHC isoforms with likely different ATPase activities implies a correlation between the contractile potential of myofibroblasts within a specific tissue and MyHC expression. To our knowledge, this is the first report to directly link the presence of skeletal MyHC to myofibroblast contraction.

The fact that skeletal MyHC function correlates with ASMA expression in myofibroblasts implies that the regulation and function of these two contractile molecules are somehow coupled. Supporting this hypothesis, we demonstrate that inhibiting contraction by treatment with BTS results in a concomitant reduction in ASMA promoter activity and protein expression in pulmonary myofibroblasts. Such results are not surprising given that the ASMA promoter can be mechanotranscriptionally regulated (Wang et al., 2002), and inhibition of myofibroblast contraction by the ASMA amino-terminal peptide, Ac-EEED, results in a concomitant reduction in ASMA mRNA (Hinz et al., 2002). The fact that inhibition of ASMA expression blocks myofibroblast contraction (Feugate et al., 2002) might imply that the observed decrease in myofibroblast contractility in the presence of BTS is due entirely to decreased ASMA expression and not MyHC per se. We do not think this is the case, as we find that the percentage of ASMA-expressing cells is the same under normal growth conditions, conditions that are highly contractile, or in the presence of inhibitor. It is reasonable to speculate instead that MyHC-mediated contractility is somehow coupled to the incorporation of ASMA into stress fibers, thus leading to a more differentiated myofibroblast. Such supposition is supported by the fact that the anticontractile effect of the Ac-EEED peptide is fast, within 30 min, which is probably not due to ASMA expression but to release of ASMA from stress fibers (Hinz et

al., 2002). The observation that transmission of externally applied forces to contractile cells, such as myofibroblasts, is linked to specific patterns of gene expression suggests that other structural proteins in these cells, e.g., MyHC, may also be transcriptionally regulated mechanically. Indeed, both IIa and IId MyHC promoter activities are decreased in the presence of BTS.

There are many similarities between muscle cells and myofibroblasts, yet it is an oversimplification to say these complex cells are an isotype of muscle, as many differences exist between them. One striking difference is that these cells express active MRFs and yet are resistant to cell cycle withdrawal. The regulation of MyHC gene expression in myofibroblasts is also distinct from muscle. Although many of the same transcriptional pathways are implicated in both cells types, how they regulate the various MyHC promoters differs significantly. In this work, we examined three different pathways known to be involved in conferring muscle-specific gene expression: the MRF family of transcription factors and two Ca^{2+}-regulated pathways, the CaM kinase and the calcineurin/NFAT pathways. In myofibroblasts, the MRF family of transcription factors appears to preferentially regulate the embryonic MyHC promoter, although overexpression of MyoD also has a modest effect on IId promoter activity. This is not the case in skeletal muscle cells, where the IIb MyHC promoter is most responsive to MyoD overexpression (Allen et al., 2001). Although myf-5 is expressed in myofibroblasts, its overexpression in these cells does not increase the activity of any of the promoter constructs tested, suggesting that saturating levels of myf-5 may already be present. Although MyoD is able to activate both the embryonic and IId MyHC promoters, it is not found in these cells, implying that myf-5 is likely the primary MRF responsible for E-box binding and promoter activation in pulmonary myofibroblasts. The expression of MRFs in pulmonary myofibroblasts is consistent with other reports of their presence in nonmuscle tissues. The expression of myf-5 has been found in embryonic brain sections (Tajbakhsh and Buckingham, 1995) and embryonic lung (Buckingham, M.E., personal communication), where pulmonary myofibroblasts are essential for alveolar development and pulmonary septation (Bostrom et al., 1996); induction of pulmonary fibrosis in mice by bleomycin treatment results in an up-regulation of myogenin gene expression (Kaminski et al., 2000), and MyoD is expressed in a number of fetal organs, including kidney, liver, and lung (Gerhart et al., 2001).

MEF2c overexpression in myofibroblasts resulted in a differential regulation of the IIa (inhibitory) and IId (activating) MyHC promoters and had little effect on the embryonic promoter. This pattern of regulation is not observed in muscle cells, where the presence of MEF2c increases the activity of all three adult fast MyHC promoters (Allen et al., 2001). Conversely, the calcineurin pathway preferentially activates the MyHC IIa promoter activity in muscle (Allen et al., 2001) yet up-regulates the activity of all three MyHC promoters in myofibroblasts. These results are further corroborated at the protein level by the observed decrease in the number of myofibroblasts expressing myosin after treatment with the calcineurin inhibitor CsA. Further delineating how Ca^{2+} signals are transduced in myofibroblasts will be important in understanding the activation and function of these cells.

Taken together, our results support an intricate cross-talk among three pathways of transcriptional regulators, the MRFs, the MEF family, and the calcineurin/NFAT pathway, in the control of MyHC expression in myofibroblasts that is different than that observed in muscle. It is intriguing that the same MyHC promoters behave differently in muscle cells versus myofibroblasts and suggests that cell context must be central in their regulation. Studies of the expression of the ASMA gene in multiple species, including rat and human, have demonstrated the importance of cell context in determining promoter elements that function in transcriptional control (Carroll et al., 1988; Min et al., 1990; Nakano et al., 1991; Blank et al., 1992). Determining those cis elements and trans factors that confer cell type–specific expression of MyHC will be key in understanding the role of these cells in various fibrotic pathologies.

Materials and methods

Reagents

All tissue culture media and materials were purchased from GIBCO BRL. Myosin inhibitors BTS and BDM were from Sigma-Aldrich. CsA was from Sandooz Research Institute.

Preparation of pulmonary fibroblasts

Pulmonary fibroblasts were isolated from 6–7-wk-old Lewis rats, as previously described (McIntosh et al., 1994), and cultured in DME containing 10% FBS, 25 μM Hepes, pH 7.4, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells used in this study were between cell passages three and eight after primary culture. It is well established that isolated fibroblasts cultured on a plastic substrate display a myofibroblast phenotype as determined by ASMA expression (Grinnell, 1994; Phan, 2002).

Contraction assays

Myofibroblasts and VICs, a gift from K. Anseth (University of Colorado at Boulder), were harvested from a monolayer culture with 0.25% trypsin and 1 mM EDTA. Trypsin was neutralized with 10% serum containing medium. Cells were seeded at 10^5 cells/reaction in collagen matrices prepared from Vitrogen 100 collagen (Celltrix Laboratories), 2× DME, and the appropriate experimental concentration of BDM, BTS, or CsA as indicated below. Aliquots (700 μl) of the cell–collagen mixtures were prewarmed to 37°C for 3–4 min and then pipetted into 24-well culture plates. After polymerization at 37°C for 1 h, 1.0 ml of DME media containing either 10% FBS, 10% FBS plus 15 mM BDM, 10% FBS plus 10 μM BTS, or 10% FBS plus CsA was added to each matrix. Matrices were cultured between 1 and 5 d (VICs) to allow the development of mechanical stress. To initiate contraction, matrices were gently released from the underlying culture dish with a spatula. Contraction was measured every hour for 6 h and then again at 18 h after contraction initiation. Only data from the 18-h time points are shown. All experiments were performed in triplicate, and every experiment was repeated a minimum of two to three times.

Immunohistochemistry

Myofibroblasts grown on glass coverslips were fixed and permeabilized with 70% acetone/30% methanol and subsequently blocked with 10% normal goat serum. Coverslips were incubated with either F59, a mAb that recognizes all isoforms of skeletal MyHC (Miller et al., 1989), a MyHC IId M-chain–specific mAb, a myf-5–specific mAb (Santa Cruz Biotechnology, Inc.), or an ASMA mAb (ICN Biomedicals). Antibody complexes were detected using IgG conjugated with either FITC or Texas red and visualized by fluorescence microscopy. For in vivo staining, 1-dpn mice were anesthetized and killed. The lungs were removed, washed with PBS, fixed with 4% paraformaldehyde, and frozen in OCT embedding media. Sections

were cut with a cryostat at 40 μm and stained as described above. Digital microscopic images were visualized and prepared using OpenLab and Adobe Photoshop® software.

RT-PCR

RNA was isolated from myofibroblasts with Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's specification. A cDNA library was prepared from the isolated RNA using SuperScript II (GIBCO BRL) reverse transcriptase and oligo dT primers. PCR was performed using MyHC isoform-specific primers designed to anneal to the UTR regions of each rat isoform. All primer sequences are provided in the supplemental information (available at http://www.jcb.org/cgi/content/full/jcb.200303194/DC1). PCR products were semiquantified by normalizing individual isoform levels to an endogenous myosin internal control that was simultaneously amplified using primers that recognize all skeletal myosin isoforms.

Proliferation assays

Myofibroblasts were seeded in six-well culture dishes at a density of 4×10^4 cells/well. Cells were grown in DME containing either 10% FBS or 1% FBS plus or minus 15 mM BDM or 10 μM BTS. Every 24 h, cells were harvested and optical density measurements were taken at 595 nm.

Plasmid promoter and overexpression constructs

The mouse IIa, IId, and IIb MyHC promoter reporter constructs have been described previously (Allen et al., 2001). The rat ASMA promoter, −713 to +52 bp, was engineered by PCR, amplifying the promoter sequence from rat genomic DNA using the primers 5′-GGACGCGTACGGTCCTTAAGCATG-3′ (forward) and 5′-GGCTCGAGCTTACCCTGATGGCG-3′ (reverse), and subsequent cloning into the pGL3basic vector (Promega). The sequence was confirmed by restriction analysis and dideoxy sequencing. The Rous sarcoma virus–MyoD expression vector was a gift of H. Weintraub (Fred Hutchison Cancer Center, Seattle, WA); the cytomegalovirus (CMV)–myf-5 expression vector was a gift of S. Konieczny (Purdue University, Lafayette, IN); and the CMV–MEF-2C, CMV–cn-NFAT, CMV–ca-calcineurin expression vectors were all provided by E. Olson (University of Texas, Dallas, TX).

Myofibroblast transfection and promoter activity reporter assays

Primary pulmonary myofibroblasts were transfected with LipofectAMINE 2000 transfection reagent according to the manufacturer's protocol. In all experiments, a thymidine kinase–*Renilla* luciferase construct (Promega) was used as an internal standard for transfection efficiency. For cotransfection studies, MyHC reporter vectors were transfected 1:1 with the expression vectors tested. After transfections, cells were assayed for both firefly (MyHC promoter constructs) and *Renilla* (internal control) luciferase activities using the commercially available dual luciferase kit (Promega). For MRF and MEF sensor plasmids, either CAT activity was measured, as previously described (Mayer and Leinwand, 1997), or β-galactosidase activity was determined using a luminescent substrate from Tropix according to the manufacturer's directions.

Western blotting

Whole cell lysates were prepared from myofibroblasts by sonication in lysis buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 0.5% NP-40). Protein concentrations were determined using the Bio-Rad Laboratories DC reagent, and 5 μg of protein was separated on a 10% SDS–polyacrylamide gel before transferring to PVDF membrane. Westerns were probed with an ASMA monoclonal antibody (ICN Biomedicals) and an alkaline phosphatase–conjugated secondary antibody (Southern Biotechnology Associates, Inc.). Blots were visualized using ECL substrate (Amersham Biosciences) and quantified using phosphorimager analysis and ImageQuant software.

Online supplemental material

The supplemental material (primer sequences) is available at http://www.jcb.org/cgi/content/full/jcb.200303194/DC1.

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