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Reversible Differentiation of Myofibroblasts by MyoD

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Abstract

Myofibroblasts participate in tissue repair processes in diverse mammalian organ systems. The deactivation of myofibroblasts is critical for termination of the reparative response and restoration of tissue structure and function. The current paradigm on normal tissue repair is the apoptotic clearance of terminally differentiated myofibroblasts; while, the accumulation of activated myofibroblasts is associated with progressive human fibrotic disorders. The capacity of myofibroblasts to undergo de-differentiation as a potential mechanism for myofibroblast deactivation has not been examined. In this report, we have uncovered a role for MyoD in the induction of myofibroblast differentiation by transforming growth factor- β 1 (TGF- β 1). Myofibroblasts demonstrate the capacity for de-differentiation and proliferation by modulation of endogenous levels of MyoD. We propose a model of reciprocal signaling between TGF- β 1/ALK5/MyoD and mitogen(s)/ERK-MAPK/CDKs that regulate myofibroblast differentiation and dedifferentiation, respectively. Our studies provide the first evidence for MyoD in controlling myofibroblast activation and deactivation. Restricted capacity for dedifferentiation of myofibroblasts may underlie the progressive nature of recalcitrant human fibrotic disorders.

Keywords

de-differentiation; fibroblasts; tissue repair; fibrosis; MyoD

INTRODUCTION

Myofibroblasts are multifunctional cells derived from mesenchymal progenitors (fibroblasts), which participate in a diverse range of physiological and pathological processes throughout the body, including wound healing and fibrosis [1–2]. In response to tissue injury, fibroblasts migrate to the site of damage and differentiate into myofibroblasts, “muscle-like” contractile cells characterized by *de novo* synthesis of alpha smooth muscle actin (α -SMA) and stress fiber formation. Myofibroblast differentiation represents a critical and necessary step of normal tissue repair and wound healing; myofibroblasts are responsible for synthesis of extracellular matrix (ECM) components which contribute to matrix reconstitution and contraction to promote wound closure [3]. As wound healing culminates, myofibroblasts are thought to undergo apoptosis to restore tissue homeostasis,

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whereas myofibroblast accumulation and activation are a pathological hallmark of fibrosis [4]. The mechanism(s) of myofibroblast deactivation and the potential for de-differentiation of myofibroblasts are largely unknown.

Despite critical roles of myofibroblasts in both wound healing and fibrosis, the mechanisms mediating myofibroblast commitment and the factors maintaining the stability of the myofibroblast phenotype remain poorly understood. Furthermore, it is unclear whether the myofibroblast represents a terminally differentiated cell type [5–6]. Elucidating the molecular pathways regulating fibroblast-to-myofibroblast differentiation and myofibroblast stability/persistence are crucial to distinguish mechanisms common to normal wound repair from those specific to pathological conditions; this understanding could lead to the development of anti-fibrotic therapeutic approaches targeting myofibroblast persistence.

MyoD is a basic helix-loop-helix (bHLH) transcription factor which plays a critical role in myogenic differentiation [7–8]. Expression of MyoD represents one of the earliest events of myogenic commitment and initiates the irreversible transition of myogenic precursors to differentiated skeletal muscle cells. MyoD activates the transcription of over 300 genes that affect various cellular functions including cell cycle/DNA replication, adhesion/matrix, metabolism, structural/cytoskeletal, and apoptosis [9]. The expression of MyoD has also been associated with myofibroblast presence in tissue repair/fibrosis [10–11]; however, the precise function of MyoD in myofibroblast differentiation remains unclear. In this study, we demonstrate an essential role for MyoD in TGF- β 1-induced myofibroblast differentiation. Additionally, we provide evidence for myofibroblast de-differentiation, which is associated with mitogen-activated protein kinase (MAPK) signaling and MyoD downregulation. These results support the concept that myofibroblasts are not terminal differentiated and that myofibroblast de-differentiation may be critical for normal regenerative vs. fibrotic responses to tissue injury.

MATERIALS AND METHODS

Reagents

Porcine platelet-derived TGF- β 1 was obtained from R&D Systems, Minneapolis, MN. Rabbit polyclonal antibody against human MyoD was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse monoclonal antibodies to α -smooth muscle actin (α -SMA), was obtained from Sigma, St. Louis, MO. Rabbit monoclonal antibody against GAPDH was from Abcam. Secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from Pierce, Rockford, IL. PD98059, SB203580, SB431542, and SP600125 inhibitors were purchased from TOCRIS Bioscience, Avonmouth, UK. Roscovitine was purchased from Cell Signaling, Danvers MA. All other reagents were obtained from Sigma.

Cell culture

We obtained human fetal lung fibroblast cells (hFLMCs; IMR-90 cells) from Coriell Cell Repositories, Institute for Medical Research, Camden, NJ. Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1.25 μ g/ml amphotericin B, and incubated cells at 37°C in 5% CO₂, 95% air.

Western immunoblotting

We prepared cell lysates in RIPA buffer, subjected them to SDS-PAGE under reducing conditions and performed western immunoblotting as previously described[12].

Densitometric analyses of Western blots were performed using the public domain NIH Image program available on the internet at www.rsb.info.nih.gov/nih-image.

Proliferation: BrdU incorporation assay and coulter counting

We serum-starved cells cultured in 96-well plates for 24h followed by BrdU pulse in media with/without 10% serum for 24 hours. We measured BrdU incorporation using a kit from Calbiochem (Cat #QIA58). We measured cell counts using a coulter counter (model ZM, Coulter Electronic, Hialeah, FL).

Immunofluorescence

Cultured cells were then fixed in 4% Formaldehyde and then permeabilized with 1% Triton-X100 in PBS. The cells were then blocked with 1% BSA in PBS to block non-specific binding. Cells were then incubated with mouse monoclonal α -smooth muscle actin and rabbit polyclonal Ki-67 antibodies (1:40 in PBS) at room temperature for 1h. The cells were then washed with PBS followed by incubation with FITC conjugated goat anti mouse IgG and TRITC conjugated goat anti rabbit IgG (1:50 in PBS) for 1h. The cells were then washed with PBS and nuclear staining with accomplished with mounting media containing DAPI. Cells were visualized with a fluorescent microscope and images were obtained.

Generation of stably over-expressing MyoD *sh*RNA and scrambled *sh*RNA mutant cell lines

A DNA based stable knockdown system p*Silencer*TM 2.1-U6 hygro from Ambion expressing a scrambled knockdown sequence that does not target known genes in mouse, rat or human transcriptome was used. The silencing cassette segment with a MCS for inserting short hair-pin coding silencing sequence specific to the gene of interest (approx 400 bp) with a U6 promoter driving a short hair-pin RNA (non targeting sequence) was excised using Hind-III and EcoRI restriction enzymes. This fragment was then sub-cloned into the MCS of pc DNA 3.1(-) plasmid.

For silencing MyoD expression, the cDNA sequence was analyzed for best possible targets spanning the entire length of the c-DNA for silencing by using online algorithm provided by Ambion and 2 random sequences of varying lengths were chosen (http://www.ambion.com/techlib/misc/siRNA_finder.html). Next, using these sequences, sense strand oligo-nucleotides were constructed with self-annealing arms around a central “hair pin loop” consisting of 6 nucleotides (*TTCAAG*) and with a BamHI restriction site at the 5' terminus and 3' RNA pol III terminator followed by a HindIII restriction site. Complimentary sequence was then generated to the sense strand. A mixture of 1:1 sense:antisense oligo-nucleotide mix was denatured to 94°C for 5 minutes in a PCR machine and allowed to anneal at 37°C for 1 hour. The resulting annealed mixture was then ligated into an open pcDNA 3.1 in place of the non-targeting sequence.

Clone 1 sense strand oligo-nucleotide sequence was as follows for targeting the region 294–312 of the MyoD *mRNA*. 5'- ATCCGCAACGGACGACTTCTATG-TTCAAG-GATCATAGAAGTCGTCCGTTGCTTTTTTGAAAA-3'

Clone 2 sense strand oligo-nucleotide sequence was as follows for targeting the region 1565–1584 of the MyoD *mRNA*. 5'-GATCCAAATGTAGCAGGTGTAACCGT-TTCAAG-AGAACGGTTACACCTGCTACATTTTTTTTGAAAA-3' The underlined sequence represents the *mRNA* annealing segment of the hair pin loop. The plasmids were then transformed into TOP-10 bacterial host and amplified. Plasmids were then sequenced to confirm successful sub-cloning.

Low passage (6–9) fibroblasts were transfected using Qiagen-Effectine reagent according to the manufacture protocol. Stable selection of transfected cells was accomplished by culturing cells with Geneticin (G-418) 350µg/ml for 48h, followed by a second incubation with 25–50µg/ml of G-418 for an additional 4–5 days. For subsequent experiments, 5µg/ml was added to the culture media. Mutant cell lines were analyzed by SDS-PAGE and western blot analysis to confirm expression of MyoD.

Statistical analysis

We expressed data from various groups as mean $\pm$ S.E.M. We made statistical comparisons using the Student's *t* test for unpaired samples.

RESULTS

TGF- β 1 induces stable myofibroblast differentiation independent of autocrine ALK5 signaling

Previous studies have shown that fibroblastic cells from a variety of tissue sources differentiate into myofibroblasts in response to TGF- β 1 [13–14]. We first determined if TGF- β 1-induced myofibroblast differentiation represents a stable and durable cellular response. We utilized human lung fibroblast cells (IMR-90s) as a model of myofibroblast differentiation [14–15]. TGF- β 1-induced fibroblast-to-myofibroblast differentiation was demonstrated by the upregulation α -SMA protein expression in a time-dependent manner and remained stable up to 4 days after a single dose of TGF- β 1 (Figure 1A). The upregulation of α -SMA protein was associated with the formation of polymerized α -actin stress fibers, as shown by immunofluorescent labeling of α -SMA (Figure 1B, first two panels).

We assessed whether the stable expression of α -SMA is dependent on autocrine effects of TGF- β 1. Despite the removal of cell culture media 16 h after initial TGF- β 1 treatment, cells continued to express α -SMA-containing stress fibers up to 48 h (Figure 1B, last two panels). To confirm the stability of the myofibroblast phenotype, we tested effects of the TGF- β type I receptor (ALK5) inhibitor, SB431542, on cells pre- and post-differentiation. Pre-treatment of cells with SB431542 (0.5 µM) completely blocks TGF- β 1-induced expression of α -SMA, as shown by Western immunoblotting (Figure 1C). Interestingly, the addition of the same ALK5 inhibitor (SB431542; 0.5 µM) at 24 or 48 h post-TGF- β 1 stimulation did not alter the expression of α -SMA when analyzed at 48 h and 72 h (Figure 1D). These data suggest that TGF- β 1 via ALK5 activation induces stable myofibroblast differentiation, which is not dependent on autocrine TGF- β 1/ALK5 signaling.

Myofibroblast differentiation by TGF- β 1 is dependent on MyoD

MyoD is a bHLH transcription factor recognized as a master regulator of the terminal differentiation of skeletal muscle [7–8]. However, a role for MyoD in myofibroblast differentiation has not been elucidated. In association with the observed time-dependent increase in TGF- β 1-induced α -SMA expression, we noted an earlier and sustained upregulation of MyoD, as determined by Western-immunoblotting (Figures 2A and 2B). We generated two stable fibroblast cell lines that express shRNA against MyoD. Knockdown of MyoD results in abrogation of α -SMA inducibility by TGF- β 1 (Figure 2C). Furthermore, cell line A that has more complete knockdown of MyoD shows almost no expression of α -SMA, while cell line B that expresses an intermediate level of MyoD maintains an intermediate capacity for α -SMA induction (Figure 2C). These data support a critical role for MyoD in the TGF- β 1-induced program of myofibroblast differentiation.

Stably differentiated myofibroblasts maintain the capacity for proliferation

Myofibroblasts are thought to be terminally differentiated cells that, by definition, have lost their ability to proliferate [5]. We assessed the proliferative capacity of fibroblasts and myofibroblasts in response to serum. Fibroblasts demonstrate robust proliferative responses to serum, while myofibroblasts also proliferate, albeit to a lesser degree (Figures 3A, cell number and 3B, BrdU incorporation). Analysis of individual cells by immunofluorescent labeling confirmed that differentiated α -SMA-expressing myofibroblasts exhibit positive Ki67 staining. These data indicate that myofibroblasts are not terminally differentiated cells. Interestingly, these proliferative myofibroblasts appear to show early signs of stress fiber dissolution in response to serum, as suggested by more diffuse staining of α -SMA (Figure 3C). The findings of retained capacity for proliferation and the reduction in stress fiber formation suggest that myofibroblasts are capable of de-differentiation.

Downregulation of MyoD potentiates cellular proliferation

Based on the observations that MyoD is required for myofibroblast differentiation and the reduction of α -SMA stress fibers in response to serum, we hypothesized that serum stimulation may downregulate MyoD expression. Stably differentiated myofibroblasts stimulated with serum over a period of five days show a steady decline in the level of α -SMA protein that correlates with downregulation of MyoD (Figures 4A and 4B). Under these conditions, when myofibroblasts appear to de-differentiate, the proliferative capacity is restored to that observed in undifferentiated fibroblasts (Figures 4C and 4D). To corroborate these data with genetic approaches, we employed an RNAi approach to knockdown myofibroblast expression of MyoD in order to examine proliferative responses. Similar to the effects seen with serum-stimulated downregulation of MyoD, RNAi-mediated MyoD knockdown resulted in increased proliferative responses (Figures 4E and 4F).

Mitogenic signaling via the ERK-MAPK pathway mediates MyoD suppression

Mitogens signal via protein kinase cascades that include the mitogen-activated protein kinases (MAPKs). To determine if specific MAPKs were involved with the downregulation of MyoD, we pre-treated cells with pharmacologic inhibitors of the classical MAPKs and examined the effect on mitogen-induced downregulation of MyoD and α -SMA expression. We first confirmed the effect of a specific mitogenic growth factor, PDGF (instead of serum), on suppression of MyoD and α -SMA in differentiated myofibroblasts. PDGF induced a downregulation of both MyoD and α -SMA, consistent with myofibroblast de-differentiation (Figure 5A–C). The effect on PDGF-induced MyoD suppression was blocked by inhibitors of ERK-1/2 (PD98059, 20 μ M), p38 (SB203580, 6 μ M), and JNK (SP600125, 1 μ M) MAPKs, and appears to be even induced by an inhibitor of cyclin-dependent kinases (CDKs; roscovitine, 10 nM) (Figures 5A and 5B). Interestingly, the effect on α -SMA downregulation was more specific to inhibition of the ERK-1/2 MAPK pathway and CDKs (Figures 5A and 5C). This suggested the possibility that myofibroblast de-differentiation by PDGF requires both the downregulation of MyoD and potentially independent effects of ERK-1/2 MAPK and CDKs on α -SMA de-stabilization and stress fiber dissolution. This was further supported by the observation that the more rapid dissolution of myofibroblast stress fibers was reversed by inhibiting these mitogenic kinases (Figure 5D). Together, these results support the role of the ERK-1/2 pathway in mediating mitogen-induced myofibroblast de-differentiation.

DISCUSSION

Cellular de-differentiation is a critical mechanism for the regenerative capacity seen in plants and amphibians [16–17]. One potential explanation for the relative lack of regenerative capacity and propensity for fibrosis in mammalian species may be the restricted

capacity for de-differentiation of terminally differentiated reparative cells. In this study, we examined the mechanisms for the differentiation of human lung fibroblasts into myofibroblasts in response to TGF- β 1, and for the de-differentiation of myofibroblasts in response to mitogenic stimuli. Our studies demonstrate that: (1) myofibroblast differentiation induced by TGF- β 1 is mediated by MyoD; (2) downregulation of endogenous MyoD regulates myofibroblast de-differentiation and proliferation; and (3) mitogen-induced downregulation of MyoD is mediated by ERK1/2 MAPK signaling.

Myofibroblasts are described as differentiated cells with features of both fibroblasts and smooth muscle cells [2, 18]. Myofibroblast differentiation is known to be mediated by TGF- β 1, in combination with matrix-adhesion [14] and biomechanical signaling [19]. Although MyoD has been implicated in smooth muscle and skeletal muscle differentiation, its role in myofibroblast differentiation has not conclusively demonstrated. Our studies using RNAi approaches provide the first evidence, to our knowledge, for an essential role of MyoD in TGF- β 1-induced myofibroblast differentiation.

MyoD is well-recognized to be a master regulator of the terminal differentiation of skeletal muscle [9]. Myofibroblasts have been considered to be a terminally differentiated cell type [5, 20]. Given our findings of the critical role of MyoD in myofibroblast differentiation, we sought to determine whether myofibroblasts are terminally differentiated by examining their capacity for de-differentiation and proliferation. Our studies show that myofibroblasts maintain capacity for proliferation, although the rates of proliferation (by both BrdU incorporation and assessment of cell numbers) in response to mitogens are reduced in comparison to undifferentiated fibroblasts. Our immunofluorescence studies indicate that this finding is not related to the responses of a small numbers of (undifferentiated) fibroblasts within a heterogeneous population. Indeed, single cells appear to dissolve their actin cytoskeletal structures before (or while) undergoing proliferation. Prolonged stimulation of myofibroblasts in serum results in a downregulation of MyoD to basal levels; under these conditions, de-differentiated cells attained almost identical proliferative capacity to that of undifferentiated fibroblasts. This is further corroborated by RNAi knockdown of MyoD which demonstrated hyper-proliferative cell responses. These studies support a previously recognized role for MyoD in inducing cell cycle arrest [21]. Although the mechanisms that mediate the downregulation of MyoD in response to serum are not entirely clear, our studies indicate a key role for ERK-MAK signaling and cyclin-dependent kinases. Together, these studies provide a model for antagonistic signaling of mitogen(s)/ERK-MAPK/CDKs vs. TGF- β 1/ALK5/MyoD (Figure 6).

Fibrotic disorders are typically associated with impairments in tissue repair/regeneration [22], and these disorders are estimated to account for up to 45% of deaths in the United States when considered in the context of multiple end-organ degenerative diseases [23]. Myofibroblasts are essential for normal tissue repair, yet the inability to terminate myofibroblast activation may underlie the progressive nature of fibrotic reactions in injured tissues. Improved understanding of the mechanisms of myofibroblast activation/deactivation will lead to more effective anti-fibrotic strategies.

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Figure 1. TGF- β 1 signaling, via the ALK5 receptor, induces stable myofibroblast differentiation
 Fibroblasts were maintained in serum-free media for 48 h and subsequently treated with/without TGF- β 1 (2 ng/ml) on day 0. (A) A time-course for α -SMA protein expression was determined by Western immunoblotting at the indicated time-points. (B) Cells were incubated with/without media replacement (serum/TGF- β 1-free media) at 16 h. Detection of α -SMA was determined by immuno-fluorescence for all conditions at 48 h. (C) Cells were pre-treated with/without pharmacologic inhibitors against ALK5 receptor kinase (SB431542; 0.5 μ M) 30 m prior to stimulation with/without TGF- β 1. α -SMA protein expression was detected by Western immunoblotting on day 2. (D) Media was replaced with TGF- β 1-free media with/without the addition pharmacologic inhibitors against ALK5 receptor kinase (SB431542; 0.5 μ M) and α -SMA protein expression was assessed at the indicated time-points.

Figure 2. MyoD is required for myofibroblast differentiation

(A–B) Fibroblasts were maintained in serum-free media for 48 h and subsequently treated with/without TGF- β 1 (2 ng/ml) on day 0. A time-course (0–48 h) of MyoD and α -SMA expression was determined by Western immunoblotting (A), and densitometric analysis (B). (C) Cells were transfected with either a non-targeting control siRNA or using RNAi strategies targeting MyoD (cell line A and B) and stable knockdown was accomplished by selection of transfected cells with geneticin. Cells were then serum starved for 48 h and treated with/without TGF- β 1 (2ng/ml) for 48 h. Expression of MyoD and α -SMA was determined by Western immunoblotting.

Figure 3. Differentiated myofibroblasts maintain the capacity for proliferation

Fibroblasts were maintained in serum-free media for 24 h and subsequently treated with/without TGF- β 1 (2 ng/ml) for 48 h to induce myofibroblast differentiation (baseline). Cells were then treated with/without 20% serum-containing medium for 72 h. Cell proliferation of was assessed by cell number using a coulter counter (A), BrdU incorporation (B), and immunofluorescence staining with Ki67 (green), α -SMA (red), and DAPI (blue) (C). Bars represent mean $\pm$ SEM; n = 4 per group.

Figure 4. Mitogenic signals induce a downregulation of MyoD and α -SMA in myofibroblasts that correlates with increased proliferative capacity

(A–B) TGF- β 1-induced myofibroblasts were treated with/without media containing 20% serum for 5 days. Expression of MyoD and α -SMA was determined by Western immunoblotting (A) and densitometric analysis (B). (C–D) Untreated fibroblasts or TGF- β 1-induced myofibroblasts were treated with/without media containing 20% serum for 2 d. Serum treated myofibroblasts were trypsinized, re-plated, and treated with/without 20% serum for an additional 5 days. Proliferation was assessed by cell number using a coulter counter (C) and BrdU incorporation (D). (E–F) Cells were transfected with either a non-targeting control siRNA or RNAi targeting MyoD, and treated with/without 20% serum for 48 h. Proliferation was assessed by cell number using a coulter counter (E) and BrdU incorporation (F). Data points on line graphs represent mean $\pm$ SEM; n = 3 per group. Bars represent mean $\pm$ SEM; n = 4–6 per group.

Figure 5. De-differentiation of myofibroblasts is mediated by the ERK-MAPK pathway that is only partially dependent on MyoD

(A–D) Cells were serum-starved for 24 h, then pre-treated for 30 m with pharmacologic inhibitors indicated, ERK-1/2 (PD98059, 20 μ M), p38 (SB203580, 6 μ M), and JNK (SP600125, 1 μ M) MAPKs, or cyclin-dependent kinases (CDKs; roscovitine, 10 nM) prior to stimulation with PDGF (50 ng/ml) for 48 h. (A–C) Cell lysates were collected and expression of MyoD and α -SMA were determined by Western immunoblotting (A) and densitometric analysis (B–C). (D) Cells were fixed for immunofluorescence staining using antibodies against α -SMA (differentiation marker), Ki67 (proliferation marker), and DAPI (nuclei). Bars represent mean $\pm$ SEM; n = 4 per group; * P < 0.05 compared to PDGF alone.

Figure 6. Proposed mechanisms of MyoD in myofibroblast differentiation and dedifferentiation
Fibroblast-to-myofibroblast differentiation is mediated by TGF-β1 ligand-induced activation of the serine-threonine kinase TGF-β type I (ALK5) receptor that signals the induction/activation of MyoD, which then mediates the upregulation of α-SMA that assembles into stress fibers in differentiated myofibroblasts. Myofibroblast dedifferentiation is activated by mitogenic factors such as PDGF that signal via tyrosine kinase receptors which activate the ERK1/2 MAPK and CDKs to downregulate MyoD and α-SMA expression. An independent effect of ERK1/2 MAPK signaling directly on stress fiber assembly/polymerization may also contribute to myofibroblast dedifferentiation.