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Role of scleraxis in mechanical stretch-mediated regulation of cardiac myofibroblast phenotype

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Roche PL, Nagalingam RS, Bagchi RA, Aroutiounova N, Belisle BM, Wigle JT, Czubryt MP. Role of scleraxis in mechanical stretch-mediated regulation of cardiac myofibroblast phenotype. Am J Physiol Cell Physiol 311: C297–C307, 2016. First published June 29, 2016; doi:10.1152/ajpcell.00333.2015.—The phenotype conversion of fibroblasts to myofibroblasts plays a key role in the pathogenesis of cardiac fibrosis. Numerous triggers of this conversion process have been identified, including plating of cells on solid substrates, cytokines such as transforming growth factor-β, and mechanical stretch; however, the underlying mechanisms remain incompletely defined. Recent studies from our laboratory revealed that the transcription factor scleraxis is a key regulator of cardiac fibroblast phenotype and extracellular matrix expression. Here we report that mechanical stretch induces type I collagen expression and morphological changes indicative of cardiac myofibroblast conversion, as well as scleraxis expression via activation of the scleraxis promoter. Scleraxis causes phenotypic changes similar to stretch, and the effect of stretch is attenuated in scleraxis null cells. Scleraxis was also sufficient to upregulate expression of vinculin and F-actin, to induce stress fiber and focal adhesion formation, and to attenuate both cell migration and proliferation, further evidence of scleraxis-mediated regulation of fibroblast to myofibroblast conversion. Together, these data confirm that scleraxis is sufficient to promote the myofibroblast phenotype and is a required effector of stretch-mediated conversion. Scleraxis may thus represent a potential target for the development of novel antifibrotic therapies aimed at inhibiting myofibroblast formation.

transcription factor; cardiac fibroblast; stretch; migration; proliferation

MYOFIBROBLASTS, AS THE ACTIVATED form of fibroblasts, are major mediators of tissue fibrosis in the heart, lungs, dermis, kidneys, and gastrointestinal tract (17, 26, 29, 36, 42). Excess deposition of fibrillar collagens in the extracellular matrix (ECM) of these tissues imparts increased stiffness and reduced organ function. Cardiac fibrosis entails a poor prognosis, negatively impacting both systolic and diastolic function and eventually leading to heart failure and death (43). There currently exists no treatment for the arrest or reversal of cardiac fibrosis (38). However, alteration of the myofibroblast phenotype may provide a novel means for the treatment and even reversal of fibrotic lesions in various tissue types (20, 21, 23, 26, 34, 37, 49).

In response to myocardial injury, the release of damage factors [such as profibrotic transforming growth factor-β (TGF-β)] and mechanical strain resulting from the loss of ECM integrity induce activation of cardiac fibroblasts and subsequent conversion to the myofibroblast phenotype (11, 14, 22, 25, 32). Cardiac myofibroblasts are characterized by hypopresentation of fibrillar collagens type I and III, increased expression of α-smooth muscle actin (α-SMA), increased adhesions and cell size, reduced proliferation and migration, and the formation of stress fibers (12, 18, 39, 44, 46). The morphological and functional changes that cardiac fibroblasts undergo during their conversion to myofibroblasts are critical to the wound healing process following myocardial injury. The localization of myofibroblasts in the infarct region and their contribution of collagen to scar formation require a decrease in their migration and proliferation, as well as increased adhesion to the surrounding matrix. Stress fibers formed during the conversion of fibroblasts to myofibroblasts impart contractility to these cells, a necessary function of scar formation and maturation (44).

The transcription factor scleraxis is necessary for proper ECM composition in collagen-rich tissues including tendons and heart valves (4, 5, 31). Mechanical stretch of tendons, tenocytes, and mesenchymal stem cells increases expression of scleraxis, as well as type I and III collagens (9, 40, 41, 48). Mechanical stretch of cardiac fibroblasts induces increased expression of type I and III collagens, possibly via a mechanism that requires protein kinase C or tyrosine kinase activity, but the effect on scleraxis expression was not determined (6, 8, 28).

We previously reported a significant increase in scleraxis in the infarct scar of rat hearts 4 wk post-myocardial infarction. This increase in scleraxis is concomitant with increased levels of fibrillar collagens, ostensibly due to direct transactivation of the collagen Iα2 promoter (1, 16). Studies by our laboratory in primary rat cardiac fibroblasts have shown that scleraxis expression is increased with TGF-β treatment, as well as during conversion to the myofibroblast phenotype induced by cell passaging, which has been shown to involve an intermediate phenotype termed the proto-myofibroblast (1, 25, 44). Most recently, we have demonstrated that scleraxis is both sufficient and required for fibroblast to myofibroblast phenotype conversion in response to TGF-β and that scleraxis regulates fibro-
blast number in the heart (3). Here we have extended these studies to determine the effect of cyclical mechanical stretch on myocardial characteristics and scleraxis expression in cardiac proto-myofibroblasts, as well as the effect of scleraxis expression on myofibroblast functions (stress fiber formation, migration, cell cycle progression, and proliferation). We found that mechanical stretch and scleraxis induce similar phenotypic changes in primary cardiac proto-myofibroblasts indicative of conversion to myofibroblasts. Furthermore, stretch induces scleraxis expression, and scleraxis is required for stretch to induce the phenotypic changes indicative of phenotype conversion, demonstrating that scleraxis plays a key role in stretch-mediated conversion of cardiac fibroblasts to myofibroblasts.

**MATERIALS AND METHODS**

**Cell culture and treatments.** Primary cardiac fibroblasts were isolated from adult male Sprague-Dawley rats using enzymatic digestion as described previously (1, 16). Freshly isolated cardiac fibroblasts (P0) were used 72 h postisolation or passed to proto-myofibroblasts (P1) or myofibroblasts (P2) for experiments. Cells were synchronized in serum-free DMEM/F12 medium including 0.1% ascorbic acid and 1% penicillin/streptomycin (GIBCO) for 24 h before infection with adenoviral constructs. P1 and P2 cells were infected with adenovirus encoding green fluorescent protein (GFP) (AdGFP) or Scleraxis (AdScx) for 24 h at multiplicity of infection (MOI) 10 or with adenoviral constructs encoding short-hairpin RNA for LacZ (AdshLacZ) or scleraxis (AdshScx) for 48 h at MOI 200; we routinely obtain infection efficiency >90% using this approach and amplified and titered adenoviruses as previously reported (1, 3, 16). Isolation of primary cardiac fibroblasts from scleraxis knockout mice or C57BL6 wild-type controls was as previously described (3). Animals were handled according to the guidelines of the Canadian Council on Animal Care; animal usage was approved by the Animal Care Committee of the University of Manitoba.

**Luciferase assay.** NIH-3T3 fibroblasts (ATCC) were transfected with the 1,500 bp-scleraxis proximal promoter (250 ng) (pGL3.10-SCX1500; GeneCopoeia) for 24 h and subjected to cyclic stretch (see below). Five nanograms of Renilla expression vector were used as transfection control. Transfections were performed with Lipofectamine 2000 (3:1) and Opti-MEM (Life Technologies). Luciferase transfection control. Transfections were performed with Lipo.

**Immunofluorescence staining.** Primary cardiac fibroblasts were plated on glass coverslips, serum-starved for 24 h, and infected with adenovirus encoding green fluorescent protein (GFP) (AdGFP) or Scleraxis (AdScx) for 24 h at multiplicity of infection (MOI) 10 or with adenoviral constructs encoding short-hairpin RNA for LacZ (AdshLacZ) or scleraxis (AdshScx) for 48 h at MOI 200; we routinely obtain infection efficiency >90% using this approach and amplified and titered adenoviruses as previously reported (1, 3, 16). Isolation of primary cardiac fibroblasts from scleraxis knockout mice or C57BL6 wild-type controls was as previously described (3). Animals were handled according to the guidelines of the Canadian Council on Animal Care; animal usage was approved by the Animal Care Committee of the University of Manitoba.

**Table 1. Primers used in one-step quantitative PCR analysis**

<table>
<thead>
<tr>
<th>Amplicon/Direction</th>
<th>Primer Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Smooth muscle actin</td>
<td>Forward: CGGGCTTTGCTGTTGATGCTGCTAGGAA</td>
</tr>
<tr>
<td>Collagen 1A1</td>
<td>Forward: TGCTCCCTTTGAGGGGGCA</td>
</tr>
<tr>
<td>Collagen 1A2</td>
<td>Forward: GTCCCAGAGACAGAGAT</td>
</tr>
<tr>
<td>Collagen 3A1</td>
<td>Forward: GGTTCCTCTTACCCCTGCTCCTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: TCTGCCACTACCTTGCATGG</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Forward: AACAGCGCCTTCTACTGGGCTGTG</td>
</tr>
</tbody>
</table>

Primers are specific for both rat and mouse transcripts.

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wounds (0 h) and wounded area 24 h later were captured using a Nikon CoolPix 995 digital camera with a ×40 objective on an inverted microscope.

Fluorescence-activated cell sorting analysis. Freshly isolated fibroblasts (P0), proto-myofibroblasts (P1), and myofibroblasts (P2) were incubated with 5-ethynyl-2′-deoxyuridine (EdU)-supplemented media for 2 h and processed for cell cycle analysis using flow cytometry and commercially available reagents (Life Technologies). Cells were fixed and permeabilized using saponin-based reagent (Life Technologies). Nuclei were stained using propidium iodide (Life Technologies, Canada) containing 0.5 mg/ml RNase A (Invitrogen) to eliminate double-stranded RNA. Data were collected using a FACSCanto flow cytometer (Becton Dickinson) and analyzed using FlowJo software.

Proliferation assay. Primary rat cardiac proto-myofibroblasts were serum starved for 24 h and then infected with AdScx or AdLacZ (control) adenoviral constructs for 24 h followed by incubation in serum-supplemented media with 10 μM EdU for another 24 h. Cells were then fixed (3.7% formaldehyde), permeabilized (0.5% Triton-X), and EdU label was detected using a commercially available kit as per manufacturer’s instructions (Life Technologies). Hoechst 33342 was used to stain nuclei. Images were acquired on a Zeiss epifluorescence microscope with a ×20 objective (Zeiss). The percentage of EdU+ cells/total cells was counted from three to four fields for each sample and calculated to represent percent cell proliferation.

Statistical analysis. Experiments were repeated with a minimum of three independent biological replicates (n = 3), such that each replicate was produced from a different rat or mouse (for primary cells). Data were analyzed by Student’s t-test or one-way ANOVA with Student-Newman-Keuls post hoc analysis. Results with P < 0.05 were considered statistically significant.

RESULTS

Mechanical stretch induces expression of fibrillar collagens and the myofibroblast marker α-SMA. Mechanical stretch has previously been reported to promote the development of myofibroblast characteristics such as increased collagen expression and stress fiber remodeling (6, 8, 27, 28, 47). To confirm a similar effect in the heart, primary rat cardiac proto-myofibroblasts were exposed to 24 h of cyclic, equibiaxial stretch (15% elongation, 1 Hz frequency) on fibronectin-coated flexible silicon membranes. mRNA levels of fibrillar collagens I and III and the myofibroblast marker α-SMA were assessed by quantitative real-time PCR. In response to stretch, mRNA levels of both type I collagen isoforms (α1 and α2) and collagen 3α1 were significantly increased compared with nonstretched but identically plated control proto-myofibroblasts (Fig. 1A). In addition, the level of α-SMA mRNA was dramatically increased by stretch more than fivefold (Fig. 1B). Similarly, Western blotting revealed a significant increase in both the 70- and 130-kDa isoforms of type I collagen (Fig. 1C). These gene expression changes are consistent with phenotype conversion to myofibroblasts.

Scleraxis promotes cardiac proto-myofibroblast phenotype conversion. To determine whether scleraxis was able to induce phenotype changes similar to mechanical stretch, we infected primary cardiac proto-myofibroblasts with a scleraxis-encoding adenoviral vector (AdScx) to ensure high efficiency transfection (Fig. 2, A and B) (1, 16). Compared with infection with GFP-encoding control adenovirus (AdGFP), infection with AdScx strongly induced collagen I protein expression, similar to the effect of stretch but at even greater relative levels (Fig. 2C).

Scleraxis expression is induced by mechanical stretch. Since stretch and scleraxis overexpression exerted similar effects on myofibroblast collagen expression, we tested whether stretch was capable of inducing scleraxis expression. Using an identical protocol as employed in Fig. 1 (15% stretch for 24 h, 1 Hz), we observed that compared with nonstretched controls, mechanical stretch significantly increased levels of scleraxis mRNA more than threefold (Fig. 3A). Stretch similarly induced scleraxis protein expression (Fig. 3B). It is unclear whether these alterations represent activation of scleraxis transcription, or changes in the stability of scleraxis mRNA and/or protein. We therefore assayed the effect of stretch on transactivation of the proximal 1,500 bp of the human scleraxis promoter via luciferase reporter assay in NIH-3T3 fibroblasts. Compared with nonstretched controls, scleraxis promoter reporter gene activity was significantly increased by stretch more than threefold (Fig. 3C), indicating that increased scleraxis levels following stretch are due primarily to gene transactivation. To investigate the effect of altered stretch conditions, we subjected primary cardiac proto-myofibroblasts to 0% (nonstretched control), 10, or 15% stretch for 24 h; additionally, we subjected cells to 15% stretch for 48 h. While 15% stretch for 24 h resulted in a significant induction of both scleraxis and collagen

Fig. 1. Mechanical stretch induces cardiac proto-myofibroblast phenotype conversion. A–C, primary rat cardiac proto-myofibroblasts were subjected to 15% equibiaxial stretch (1 Hz) on flexible silicone membranes for 24 h. Stretch increased levels of mRNA for the fibrillar collagens 1α1, 1α2, and 3α1 (A), as well as the myofibroblast marker α-smooth muscle actin (α-SMA) (B). Fibrillar collagen I expression was also induced by stretch (C). Results were normalized to either GADPH (A and B) or α-tubulin (C) and control (nonstretched) cells; mean ± SE; n = 3; *P < 0.05 vs. control.

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Intriguingly, the stretch-mediated increase in cell size was dramatically attenuated in cardiac proto-myofibroblasts isolated from scleraxis knockout mice (Fig. 4C), suggesting that these cells failed to undergo stretch-induced phenotype conversion. Unstretched scleraxis knockout cells were significantly smaller than wild-type cells, and a 90% relative increase in cell size in response to stretch in wild-type cells was reduced to a ~27% increase in scleraxis knockout cells. To confirm this result, we assayed mRNA levels of collagen 1α1 and α-SMA, which are both induced by phenotype conversion to myofibroblasts. In wild-type mouse proto-myofibroblasts, stretch significantly upregulated both markers as anticipated (Fig. 4D). In stark contrast, not only were collagen 1α1 and α-SMA mRNA levels reduced in scleraxis null cells in agreement with our recently published data (3), but stretch failed to increase mRNA levels of these markers. Together, these results demonstrate that scleraxis is required for stretch-induced phenotype conversion to myofibroblasts.

Scleraxis alters adhesion and migration of cardiac proto-myofibroblasts. Decreased focal adhesion turnover in myofibroblasts concomitant with the increased presence of filamentous F-actin is indicative of increased cell-substrate adhesion (7). Phalloidin staining of cardiac proto-myofibroblasts revealed a striking increase in F-actin expression and fiber density following scleraxis overexpression compared with LacZ-infected controls (Fig. 5A). Concomitantly, there were dramatic increases in both general vinculin staining and the presence of peripherally located vinculin-positive focal adhesions (Fig. 5B), indicative of increased stable focal adhesion formation.

Increased adhesion reduces cell migration; thus we assayed the effect of scleraxis on cardiac proto-myofibroblast migration via two independent approaches. Using a wound-healing scratch assay, we observed that fewer scleraxis-infected cells migrated into the cell-denuded region at 24 h postwounding of the cell monolayer, compared with either noninfected or GFP-infected controls (Fig. 5C). In contrast, scleraxis shRNA-mediated knockdown promoted cell migration into the wound compared with a control shRNA targeting LacZ (Fig. 5C). These results were confirmed using a modified Boyden chamber Transwell migration assay, which employed platelet-derived growth factor-BB as a chemoattractant. Scleraxis overexpression reduced cell migration by more than half (Fig. 5D). Conversely, scleraxis knockdown significantly increased the number of migrated cells after 24 h by more than 50% (Fig. 5D). Scleraxis overexpression also resulted in a significant increase in the expression of integrin-β1, which contributes to the structure of stable focal adhesions (Fig. 5E), similar to the increase in integrin-β1 expression induced by cell stretch (Fig. 5F). Together, these results indicate that scleraxis overexpression promotes focal adhesion formation and stabilization and increased cell adhesion to the substratum, which in turn reduces cell migration congruent with the transition to myofibroblasts (39).

Scleraxis reduces cell cycle progression. Previous studies have shown that myofibroblasts are less proliferative than fibroblasts (46). Sequential passaging on standard stiff plastic tissue culture plates promotes fibroblast to myofibroblast conversion (15, 39). Freshly isolated (P0) cardiac fibroblasts, first passage (P1) proto-myofibroblasts, and second passage (P2)
myofibroblasts were therefore labeled with propidium iodide and analyzed via flow cytometry to assess the proportion of cells in various stages of the cell cycle. With fibroblast to myofibroblast phenotype conversion, we observed an increase in the absolute number of cells in the G0/G1 phase, a reduction in cells in S phase, and an increase in the number of cells in the G2/M phase, indicating that cells are progressively exiting the cell cycle (Fig. 6A). Normalized to total cell counts, the proportion of cells in these phases changed similarly during fibroblast to myofibroblast transition (Fig. 6B), confirming a decreased number of cycling cells.

Since scleraxis, like cell passing, promotes fibroblast to myofibroblast transition, we determined whether scleraxis exerted a similar inhibitory effect on cell proliferation. Scleraxis overexpression in cardiac proto-myofibroblasts resulted in a reduction of EdU labeling, indicating reduced proliferation (Fig. 6, C and D). Similarly, scleraxis also induced cardiac proto-myofibroblasts to exit the cell cycle as determined using flow cytometry (Fig. 6E). Indeed, cells overexpressing scleraxis exhibited a similar profile of relative cell numbers in G0/G1, S, and G2/M phases as P2 myofibroblasts (Fig. 6F). We confirmed these results by assaying proliferating cell nuclear antigen (PCNA) protein expression. PCNA expression decreased significantly with passage number as P0 fibroblasts progressed to P2 myofibroblasts (Fig. 6G). Both cell stretch (Fig. 6H) and overexpression of scleraxis (Fig. 6I) similarly reduced PCNA expression in proto-myofibroblasts to nearly the degree observed in P2 myofibroblasts. Scleraxis thus appears to be sufficient to cause cell cycle exit and reduced proliferation of cardiac proto-myofibroblasts.

DISCUSSION

Although cardiac myofibroblasts are critical for deposition of a collagen-rich matrix following myocardial infarction, their persistence in the noninfarcted and distal regions of the myocardium is the basis for cardiac fibrosis (38). Although many factors have been shown to be involved in the conversion of cardiac fibroblasts to myofibroblasts, many of these (such as TGF-β) are pleiotropic molecules that represent poor choices for therapeutic targets. We recently demonstrated that scleraxis is sufficient to induce epithelial-to-mesenchymal transition and is required for fibroblast to myofibroblast phenotype conversion induced by TGF-β, suggesting that scleraxis is a key cell phenotype regulator in these cells (3). Here we show that scleraxis is a critical regulator of the cardiac myofibroblast phenotype in response to mechanical stretch, consistent with such a role.

In vitro and in vivo studies have demonstrated the ability of mechanical tension to drive the conversion of fibroblasts to myofibroblasts (24). Here, we confirm these findings in cardiac proto-myofibroblasts, an intermediate and plastic cell type in what has been described as a continuum of cell conversion (19). Due to its intermediate nature and responsiveness to different stimuli, the proto-myofibroblast provides an ideal model to study the role of scleraxis in the myofibroblast transition, we determined whether scleraxis expression was modulated by mechanical stretch. We observed that scleraxis mRNA and protein expression were significantly increased in stretched cardiac proto-myofibroblasts compared to nonstretched controls (Fig. 6C and D). This upregulation was consistent with the observed increase in myofibroblast markers, such as collagen type I and α-SMA, in stretched cardiac proto-myofibroblasts (Fig. 1). The increased expression of scleraxis correlated with a decrease in cell proliferation, as evidenced by a decrease in EdU labeling and PCNA expression (Fig. 6E and F).

In addition to its role in myofibroblast differentiation, scleraxis also plays a critical role in the maintenance of the myofibroblast phenotype. Our results demonstrate that overexpression of scleraxis in cardiac proto-myofibroblasts resulted in a decrease in cell proliferation, as assessed by EdU labeling and PCNA expression (Fig. 6C and D). This effect was further confirmed by a decrease in luciferase expression in cells overexpressing scleraxis, suggesting that scleraxis inhibits cell proliferation. These findings are consistent with previous studies showing that scleraxis expression is increased in myofibroblasts in response to mechanical stretch (24).

In conclusion, our study provides new insights into the role of scleraxis in cardiac myofibroblast phenotype conversion. We demonstrate that scleraxis expression is increased in stretched cardiac proto-myofibroblasts, and this increase correlates with a decrease in cell proliferation. Furthermore, we show that overexpression of scleraxis in cardiac proto-myofibroblasts results in a decrease in cell proliferation, suggesting that scleraxis plays a critical role in the maintenance of the myofibroblast phenotype. These findings have important implications for the development of therapeutic strategies to target myofibroblast proliferation in the context of cardiac fibrosis.
characteristic of the myofibroblast phenotype, i.e., increased cell size and spreading (Fig. 4A). Importantly, these phenotypic changes in response to stretch were critically dependent on scleraxis function, as scleraxis null cells were significantly smaller than wild-type, exhibited attenuated cell size changes in response to stretch, and failed to upregulate collagen 1α1 and α-SMA in response to stretch (Fig. 4B, C and D), indicating that myofibroblast conversion was impaired. Although previous studies have demonstrated increased scleraxis expression in several cell types in response to cyclic stretch, we are the first to show that this phenomenon occurs in cardiac fibroblasts (Fig. 3). Our results demonstrate that scleraxis is necessary for stretch-induced cardiac myofibroblast characteristics (Fig. 7). This notion is further supported by the finding that scleraxis expression itself is able to drive a significant increase in levels of collagen 1α1 (1, 16, 30). Increased activity of the 1,500-bp scleraxis proximal promoter reporter gene in response to stretch indicates that this promoter may potentially act as an integrator of upstream signals to mediate stretch-induced cardiac myofibroblast phenotype conversion.

We have expanded on the existing literature that reported a transient increase in FAK phosphorylation at tyrosine 397 in response to 24 h of cyclic stretch followed by a decline after 6 h (13). We demonstrate here for the first time significant downregulation of tyrosine 397 phosphorylation in response to 24 h of cyclic stretch: a phenomenon implicated in both decreased focal adhesion turnover and increased cell adhesion, additional hallmarks of the conversion to the myofibroblast phenotype (10).

Our study utilized both gain- and loss-of-function experiments to demonstrate the necessity of scleraxis in regulating additional key characteristics of the cardiac myofibroblast phenotype. The conversion of myofibroblasts is marked by an
Fig. 5. Scleraxis induces stress fiber formation and attenuates cell migration. 

A: cardiac proto-myofibroblasts were infected with scleraxis (AdScx) or control (AdLacZ) adenovirus and stained for F-actin (red). Magnification: ×400; scale bar = 50 μm. 

B: cells as in A were labeled for vinculin (green) or nuclei (DAPI: blue). Vinculin-rich focal adhesions are indicated by arrowheads. Magnification: ×400; scale bar = 50 μm. Results are representative of 20 fields per biological replicate and 3 independent replicates. 

C: confluent monolayers of cardiac proto-myofibroblasts were scored with a pipette tip and visualized at 0 or 24 h. Cells were untreated (control) or infected with adenovirus encoding scleraxis (AdScx) or GFP control (AdGFP); alternatively, cells were infected with adenovirus encoding shRNA targeting scleraxis (AdshScx) or LacZ (AdshLacZ). Dashed lines mark the boundaries of the scratch. Magnification: ×100; scale bar = 100 μm. 

D: primary cardiac proto-myofibroblasts were infected with adenoviruses as in C and then subject to Transwell migration assay. The number of cells migrated in 24 h was assessed; mean ± SE; n = 3; *P < 0.05 vs. control or AdGFP (AdScx) or vs. control or AdshLacZ (AdshScx). 

E and F: integrin β1 expression was assayed in primary cardiac proto-myofibroblasts infected with AdGFP or AdScx (E) or subjected to 15% equibiaxial stretch (1 Hz) for 24 h vs. unstretched controls (F), and normalized to α-tubulin and either AdGFP or unstretched controls; mean ± SE; n = 3; *P < 0.05 vs. AdGFP or control. Note: the loading control shown in F is the same as in Fig. 6H as the blot was reprobed.
increase in cell adhesion, which is attributed to increased size and strength of focal adhesions and associated actin fibers, which mature into larger, thicker stress fibers. Colocalization of focal adhesion proteins such as vinculin at adhesion sites promotes further recruitment of focal adhesion components, as well as association with the intracellular actin cytoskeleton (27). Contractility is an important hallmark that differentiates myofibroblasts from fibroblasts, facilitating wound closure and

A

B

C

D

E

F

G

H

I

PCNA protein expression (fold)

Control Stretch

AdGFP AdScx

PCNA protein expression (fold)

AdGFP AdScx

PCNA protein expression (fold)

Control Stretch
In addition to being more synthetic, more adhesive, and less migratory, myofibroblasts are also less proliferative; this function is primarily required in the very early stages of the response to cardiac injury, wherein fibroblasts are activated and recruited to the damage site. Once established in the wounded area, however, the need for myofibroblast migration and proliferation decreases, and the primary function of myofibroblasts is to produce ECM to replace dead cardiomyocytes and necrotic tissue (33). We demonstrate that, in fact, conversion of freshly isolated (P0) cardiac fibroblasts, through the first passage (P1) proto-myofibroblast phenotype to the second passage (P2) myofibroblast phenotype, is marked by a reduction of the total number of cells in the synthetic and repair phases of the cell cycle (S and G2M; Fig. 6, A and B). In keeping with our findings that scleraxis drives the conversion to the myofibroblast phenotype, overexpression of scleraxis in proto-myofibroblasts both significantly reduces proliferation, indicated by a reduction in the number of EdU-positive cells (Fig. 6D) and decreased PCNA expression (Fig. 6I), and reduces the total number of cells in the synthetic and repair phases of the cell cycle (Fig. 6, E and F).

A key outstanding question is the delineation of exactly how scleraxis exerts its myriad effects on focal adhesion formation, migration, and proliferation. Our previous studies have greatly expanded on the number of direct target genes of scleraxis, demonstrating direct transcriptional regulation of collagen 1α2, α-SMA, Snai1, Twist1, and fibronectin (2, 3). It is possible that scleraxis similarly directly regulates genes encoding integrin-β1 and vinculin and perhaps even cyclin-dependent kinases or other regulators of cell cycling. Future studies will be required to comprehensively identify novel scleraxis gene targets as well as the mechanism by which such regulation may occur.

Our findings thus demonstrate that scleraxis is transcriptionally activated by stretch and in turn regulates the major characteristics of the myofibroblast phenotype: induction of collagen synthesis; formation of focal adhesions and stress fibers; reduction of migration; and decreased proliferation with cell cycle exit. Together, this work provides strong evidence that scleraxis is a novel regulator of the cardiac myofibroblast phenotype and paves the way for further studies examining the potential for scleraxis as a highly specific therapeutic target for the reversal of the myofibroblast phenotype and attenuation of cardiac fibrosis in vivo.

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**Fig. 6.** Scleraxis reduces cardiac proto-myofibroblast proliferation. A: freshly isolated cardiac fibroblasts (P0), proto-myofibroblasts (P1), or myofibroblasts (P2) were stained with propidium iodide and assessed for cell cycle phase by flow cytometry. B: cell cycle data from A was normalized to total counted cells. C: cardiac proto-myofibroblasts infected with adenovirus encoding GFP (AdGFP) or scleraxis (AdScx) were stained with EdU to assess cell proliferation; red stain marks EdU+ proliferating cells, blue stain (Hoechst 33342) marks all cell nuclei; magnification: ×200; scale bar = 50 μm. D: results from the experiment depicted in C were quantified; mean ± SE; n = 3; *P < 0.05 vs. AdGFP. E: AdGFP- or AdScx-infected cardiac proto-myofibroblasts were assessed for cell cycle as in A. F: cell cycle data from E was normalized to total counted cells. G: cardiac fibroblasts (P0), proto-myofibroblasts (P1), or myofibroblasts (P2) were assessed for PCNA expression by Western blot. Results were normalized to α-tubulin and P0; mean ± SE; n = 3; *P < 0.05 vs. P0; #P < 0.05 vs. P1. H: cardiac proto-myofibroblasts subjected to 15% equibiaxial stretch (1 Hz) for 24 h or unstretched controls were assessed for PCNA expression. Results were normalized to α-tubulin and unstretched controls; mean ± SE; n = 3; *P < 0.05 vs. control. Note: the loading control shown in H is the same as in Fig. 5F as the blot was reprobed. I: AdGFP- or AdScx-infected cardiac proto-myofibroblasts were assayed for PCNA expression. Results were normalized to α-tubulin and AdGFP; mean ± SE; n = 3; *P < 0.05 vs. control.

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**Fig. 7.** Model of stretch-induced scleraxis-mediated cardiac fibroblast phenotype conversion. Mechanical stretch induces scleraxis gene expression via direct transactivation of its promoter. In turn, scleraxis is required for and activates the fibroblast to myofibroblast phenotype conversion program, whose hallmarks include increased fibrosis (via direct transcriptional control of collagen gene expression) and cell contraction (via direct transcriptional control of α-SMA expression) (3). Myofibroblasts also exhibit decreased migration due to increased cell adhesion via increased expression of focal adhesion components vinculin, integrin-β1 (Itgβ1) and F-actin and reduced FAK phosphorylation at Y397, as well as decreased proliferation via an unknown mediator.
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