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Whole animal knockout of smooth muscle alpha-actin does not alter excisional wound healing or the fibroblast-to-myofibroblast transition

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Abstract

The contractile phenotype and function of myofibroblasts have been proposed to play a critical role in wound closure. It has been hypothesized smooth muscle alpha-actin expressed in myofibroblasts is critical for their formation and function. We have used smooth muscle α -actin-null mice to test this hypothesis. Full-thickness excisional wounds closed at a similar rate in smooth muscle α -actin-null and wild type mice. In addition, fibroblasts in smooth muscle α -actin-null granulation tissue when immunostained with a monoclonal antibody that recognizes all muscle actin isoforms exhibited a myofibroblast-like distribution and a stress fiber-like pattern, demonstrating that these cells acquired the myofibroblast phenotype. Dermal fibroblasts from smooth muscle α -actin-null and wild type mice formed stress fibers and supermature focal adhesions, and generated similar amounts of contractile force in response to transforming growth factor- β 1. Smooth muscle γ -actin and skeletal muscle alpha-actin were expressed in smooth muscle α -actin-null myofibroblasts, as demonstrated by immunostaining, real-time PCR, and mass spectrometry. These results demonstrate that smooth muscle α -actin is not necessary for myofibroblast formation and function and for wound closure, and that smooth muscle γ -actin and skeletal muscle α -actin may be able to functionally compensate for the lack of smooth muscle α -actin in myofibroblasts.

Keywords

wound healing; smooth muscle α -actin; myofibroblast; cytoskeleton; stress fiber; focal adhesion

INTRODUCTION

Myofibroblasts are specialized contractile fibroblasts that are proposed to play a key role in generating contractile forces responsible for wound closure and pathological contractures (1–4). These cells are characterized by the acquisition of a contractile phenotype, which includes the formation of large stress fibers and supermature focal adhesions (5–7). In addition, myofibroblasts express smooth muscle α -actin (SMA) (3, 8), an actin isoform

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found predominantly in smooth muscle cells. One of the key questions concerning myofibroblast formation and function is the role of SM α A in the acquisition of the contractile phenotype and the generation of contractile force.

There are six actin isoforms found in all mammalian cells: two cytoplasmic actin isoforms that are ubiquitously and highly expressed in non-muscle cells, cytoplasmic β -actin (CY β A) and cytoplasmic γ -actin (CY γ A), and four muscle actin isoforms that are named for their primary localization--SM α A, smooth muscle γ -actin (SM γ A), skeletal muscle α -actin (SkM α A), and cardiac muscle α -actin (CM α A) (9). SM α A makes up approximately 20% of the total actin found in myofibroblasts (10). Expression of SM α A in myofibroblasts has been correlated with the acquisition of the contractile phenotype and force generation (3, 11). In addition, increased expression of SM α A by itself is sufficient to increase stress fiber and focal adhesion assembly and increase generation of contractile force (11). These results suggest expression of SM α A in myofibroblasts plays a key role in their formation and function. However, studies have demonstrated that myofibroblasts express other smooth muscle contractile proteins which may also play an important role in myofibroblast formation and function, including SM22 α , h1-calponin, and SM γ A (12, 13). Recent studies have demonstrated that decreased expression of contractile genes with CArG elements in their promoter, including SM α A, SM γ A, SM22 α , and h1-calponin, can reduce stress fiber and focal adhesion assembly, as well as myofibroblast formation and function (13, 14). These results raise the question as to whether SM α A is necessary for myofibroblast formation and function or whether other contractile proteins could compensate for SM α A.

Previous studies have demonstrated that smooth muscle cells can still function in the absence of SM α A. SM α A-null mice are healthy and survive through adulthood, demonstrating that both vascular and visceral smooth muscle can function without SM α A, although contractile force generation is reduced in both vascular and bladder smooth muscle of SM α A-null mice (15, 16). Expression of other actin isoforms in the smooth muscle of these SM α A-null mice-- SkM α A in vascular smooth muscle cells (15) and SM γ A in bladder smooth muscle cells (16)--suggests that expression of these other actin isoforms may compensate for lack of SM α A. Interestingly, myoepithelial cell function is dramatically decreased in SM α A-null mice, suggesting that these epithelial-derived contractile cells cannot compensate due to the lack of expression of other muscle actin isoforms (17, 18).

To determine the role of SM α A in myofibroblast formation and function during wound closure, we examined closure of excisional wounds on the dorsum of SM α A-null mice. In addition, SM α A-null fibroblasts were treated with transforming growth factor- β 1 (TGF- β 1), which promotes myofibroblast formation (11, 19), and examined for their ability to acquire the myofibroblast phenotype and generate contractile force in tissue culture models of wound contraction. We found that SM α A is not necessary for excisional wound closure and that the mechanical and growth factor environment in SM α A-null wounds is sufficient to induce SM α A promoter activity. Fibroblasts in SM α A-null granulation tissue positively stained with a monoclonal antibody that recognizes all muscle actin isoforms, exhibiting a myofibroblast-like distribution and a stress fiber-like pattern, thus demonstrating that these cells acquired the myofibroblast phenotype. In addition, cultured SM α A-null fibroblasts can acquire the myofibroblast phenotype and generate contractile force similar to WT fibroblasts in response to TGF- β 1. We have also demonstrated by immunostaining, real-time PCR, and mass spectrometry that SM γ A and SkM α A are expressed in cultured SM α A-null myofibroblasts and organized into stress fibers. These results suggest that SM α A is not necessary for myofibroblast formation and function, and that other muscle actin isoforms and/or contractile proteins can compensate for its loss.

MATERIALS AND METHODS

Animals

SM α A-null mice were generated by inserting the Pol2NeobpA cassette into the +1 start site of the SM α A gene (15). SM α A-null and WT mice were genotyped as previously described (16, 17). Mice possessing a transgene composed of the SM α A promoter construct from –2600 through the first intron conjugated to the β -galactosidase reporter (p2600Int/LacZ) have been previously described (20, 21). SM α A-null mice carrying the SM α A promoter construct (*Acta2*^{–/–}/p2600Int/LacZ) were produced by breeding SM α A-null with p2600Int/LacZ animals. Genotypes were determined as previously described (16, 21). All mice were maintained and bred under standard pathogen-free conditions and all protocols were approved by the University of Oklahoma Health Sciences Institutional Animal Care and Use Committee.

Monoclonal Antibodies Used to Recognize Actin Isoforms

The following monoclonal antibodies were used to recognize different actin isoforms: all actin isoforms, clone C4 (22) (MAB1501, Millipore, Billerica, MA); all muscle actin isoforms, clone B4 (22) (LMAB-B4, Seven Hills Bioreagents, Cincinnati, OH); CY β A, clone 4C2 (23) (NBP1-97721, Novus Biologicals, Littleton, CO); CY γ A, clone 2A3 (23) (NBP1-97720, Novus Biologicals); SM α A, clone 1A4 (24) (A2547, Sigma-Aldrich, St. Louis, MO); SM α A/SM γ A, clone CGA7 (25) (A7607, Sigma-Aldrich); and SkM α A/CM α A, clone 5C5 (26) (A2172, Sigma-Aldrich). A description of which actin isoform(s) each monoclonal antibody recognizes is in Table 1.

Dermal Excisional Wounding

General anesthesia was induced, in male mice between 6–8 weeks of age, by intraperitoneal injection of avertin with 0.016 ml of 2.5% avertin/g of body weight (21). The back was shaved and full-thickness, circular, excisional wounds of 4 mm or 7 mm were made on the dorsum in the midline, with one wound per animal and left uncovered (21). Wounds were digitally photographed with an Olympus MVX10 Stereomicroscope (Olympus, Center Valley, PA) at 0, 1, 2, 3, 4, 7 days post-wounding for 4 mm diameter wounds and 0, 3, 7, 10, 14 days post-wounding for 7 mm diameter wounds. The wound area was determined using Image-Pro Plus Software (Media Cybernetics, Bethesda, MD) and expressed as percentage of initial wound size.

Histology

Mice with 7 mm diameter wounds were euthanized by CO₂ asphyxiation 12 days post-wounding. The wound plus surrounding dermis was excised and fixed in neutral buffered formalin overnight at 4°C, embedded in paraffin, sectioned, stained with hematoxylin and eosin, photographed, and the distance between the edges of the panniculus carnosus determined as a measure of wound contraction using Image-Pro Plus Software.

Expression of the p2600Int/LacZ transgene was evaluated in *Acta2*^{–/–}/p2600Int/LacZ mice with 7 mm diameter wounds 12 days post-wounding. Mice were euthanized by CO₂ asphyxiation and granulation tissue excised, fixed and stained en block for β -galactosidase activity (21). Tissues were dehydrated, paraffin-embedded, sectioned and evaluated unstained or stained with hematoxylin and eosin.

Immunohistochemistry

Mice with 7 mm diameter wounds were euthanized by CO₂ asphyxiation 12 days post-wounding and the wound plus surrounding dermis excised and fixed in 4%

paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, frozen, and cryosectioned. Cryosections underwent antigen retrieval with antigen unmasking solution (H-3300, Vector Laboratories, Burlingame, CA) for 10 minutes at 95°C. Immunostaining was performed using a M.O.M.TM Kit for detecting mouse primary antibodies on mouse tissues (BMK-2202, Vector Laboratories). To visualize all muscle actin isoforms, cryosections were incubated with clone B4 at 1:500 followed by visualization with streptavidin conjugated to Alexa Fluor 546 (S-11225, Invitrogen, Carlsbad, CA). To visualize SMA, cryosections were immunostained with clone 1A4 conjugated to Cy3 (1:400) (C6198, Sigma-Aldrich).

Cell Culture, Immunofluorescence, and Quantification of Focal Adhesions

Primary dermal fibroblasts were isolated from day 1 post-natal mice as previously described (27). Fibroblasts were cultured in complete medium composed of Dulbecco's Modified Eagle's Media (Invitrogen), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic-antimycotic solution (Sigma-Aldrich) and 1% sodium pyruvate solution (Sigma-Aldrich). For all experiments fibroblasts were used at passage 2–3. To promote myofibroblast differentiation, fibroblasts were cultured for 4 days in complete medium with 1 ng/ml TGF- β 1 (Sigma-Aldrich) (19). To visualize actin cytoskeleton or focal adhesions, myofibroblasts were fixed and stained as previously described (13, 19) using clone 1A4 (1:1000), clone B4 (1:100), clone CGA7 (1:75), clone 5C5 (1:500), anti-vinculin antibody (1:500) (V9131, Sigma-Aldrich), rhodamine-phalloidin (R415, Molecular Probes, Life Technologies, Carlsbad, CA), or 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, D1306, Molecular Probes). Fluorescence microscopy and image capture was performed using an Olympus Provis AX-70 photomicroscope (Olympus) equipped with a QImaging digital camera (QImaging, Surrey, British Columbia, Canada). Focal adhesion size was quantified by obtaining images of 10 cells per treatment and analyzing for focal adhesion size and number using Image Pro Plus Software (6).

Collagen Lattice Contraction Assays

Measurement of contractile force generated within a stress-relaxed collagen lattice was performed as previously described (19, 28, 29). Fibroblasts were cultured within stabilized type I collagen lattices (collagen concentration, 0.65 mg/ml; cell concentration, 1.25×10^5 cells/ml) in complete medium without or with 1 ng/ml TGF- β 1, with TGF- β 1 replenished at 2.5 days. After 5 days in culture, generation of contractile force was analyzed by measuring the lattice diameter before release and at specific times after release using a Nikon SMZ-1 stereoscope and the fraction of the original collagen lattice diameter was calculated. All contraction assays were carried out in triplicate, and every experiment was repeated three or more times; values represent mean of multiple experiments $\pm$ standard error of the mean (SEM).

Contractile force generation was also measured using a culture force monitor (30, 31). Fibroblasts were cultured in complete medium for 5 days without or with 1 ng/ml TGF- β 1 prior to trypsinization and placement within the collagen lattice (collagen concentration, 1 mg/ml; cell concentration, 0.5×10^6 cells/ml). A rectangular cell-populated collagen lattice was cast between a fixed point and a force transducer and cultured in complete medium without or with 1 ng/ml TGF- β 1. Cell-generated forces in the collagen lattice were detected by the force transducer and analyzed using AcqKnowledge software (Biopac System, Inc., Goleta, CA). Data were collected every second for 18 hours providing a continuous output of force (dynes: 1×10^{-5} N). Experiments were performed three independent times. A representative trace is shown for each experiment with each point on the trace an average of 1080 points.

Reverse Transcription-Polymerase Chain Reaction

SM α A levels in granulation tissue were analyzed by semi-quantitative RT-PCR. Mice with 7 mm diameter wounds were euthanized by CO₂ asphyxiation 12 days post-wounding. Granulation tissue was placed in RNAlater-Ice (Invitrogen) and thawed overnight. RNA was isolated using Trizol Reagent (Invitrogen) per manufacturer's protocol. Total RNA was quantified by spectrophotometry and reverse transcribed using Superscript III cDNA Synthesis Kit (Invitrogen) (13). Semi-quantitative RT-PCR was performed as described previously (32).

Expression of actin isoforms in fibroblasts cultured for 4 days in complete medium with 1 ng/ml TGF- β 1 was determined by real-time RT-PCR. Total RNA was extracted from cells using Trizol Reagent (Invitrogen), and RNA was reverse transcribed and used for real-time PCR analysis in a Bio-Rad iCycler (Bio-Rad, Hercules, CA) as previously described (13). The comparative cycle at threshold (Ct) method was used to determine relative changes in mRNA levels compared to control gene. Primer sequences are reported in Supplemental Table S1.

Western Blot Analysis

Expression of total actin and different actin isoforms in fibroblasts cultured for 4 days in complete medium with 1 ng/ml TGF- β 1 was determined by Western blotting. Whole cell lysates were collected in 1% SDS lysis buffer, and total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) (13). Samples were adjusted to equal protein concentration and separated by SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane (Protran BA 83, Whatman, Dassel, Germany), incubated with Blocking Buffer (Rockland Immunochemicals, Inc., Gilbertsville, PA), and incubated overnight at 4°C in Blocking Buffer with clone C4 (1:5000 dilution), clone B4 (1:500 dilution), clone 4C2 (1:1000 dilution), clone 2A3 (1:1000 dilution), clone 1A4 (1:5000 dilution), clone CGA7 (1:300 dilution), or clone 5C5 (1:2000 dilution). Blots were washed in Tris-buffered saline plus Triton X-100, then incubated at ambient temperature for 1 hour with goat anti-mouse IgG conjugated to horseradish peroxidase (1:3000; BioRad, Hercules, CA). Blots were washed, then imaged using ECL Western Blotting Substrate (Pierce, Rockford, IL) and a BioSpectrum 600 Imaging System (UVP, Upland, CA).

Statistics

Data are reported as mean $\pm$ standard deviation of at least four experiments except for stress-relaxed collagen lattice contraction analyses which are reported as mean $\pm$ standard error of the mean. Difference between means was tested using an unpaired, two-tailed, Student's *t*-test for all experiments except, for real time RT-PCR analyses which used a one-way ANOVA with Tukey's Multiple Comparison test. A value of *p* < 0.05 was considered statistically significant.

RESULTS

SM α A expression is not necessary for closure of excisional wounds in mice

To determine whether SM α A expression is necessary for wound closure in mice, 4 mm or 7 mm diameter full-thickness, circular excisional wounds were made on SM α A-null or WT mice. As expected, SM α A mRNA was present in WT dermis and increased in day 12 granulation tissue; no mRNA for SM α A was observed in SM α A-null dermis or day 12 granulation tissue (Supplemental Figure 1). No major differences were observed in macroscopic wound closure in SM α A-null compared with WT mice of 7 mm wounds (Figure 1a) or 4 mm wounds (not illustrated). Macroscopic measurement of wound closure

showed that the 4 mm wounds closed more rapidly than 7 mm wounds (compare Figures 1b and c). The 4 mm wounds on the SM α A-null mice closed significantly slower ($p<0.05$) at days 2 and 3; however, by day 4, and later, there was no difference in wound closure between SM α A-null and WT mice (Figure 1b). Wound closure of the 7 mm wounds between SM α A-null and WT mice showed no significant differences at any of the times examined, although the wounds on SM α A-null mice closed slightly slower than the WT mice at all days measured (Figure 1c). These macroscopic findings were confirmed by histological assessment of wound closure of 7 mm excisional wounds at 12 days post-wounding. No major differences in histological appearance of SM α A-null and WT were observed (Figures 1d and e; Supplemental Figure 2). Also, there was no significant difference in distance between the wound margins in 12 day SM α A-null and WT granulation tissue, although this distance was greater in SM α A-null than in WT mice (SM α A -null: 1.51 ± 0.347 ; WT: 1.24 ± 0.309 ; $n=6$). These results suggest that, while wound closure may be slightly retarded in SM α A-null mice, wound closure can occur and SM α A expression is not essential for closure of excisional wounds.

We have previously demonstrated, that the transgene containing the SM α A promoter conjugated to β -galactosidase (p2600Int/LacZ) is active in myofibroblasts in granulation tissue (21). Here, we found that fibroblasts in 12-day SM α A-null granulation tissue expressed β -galactosidase, indicative of SM α A promoter activity (Figures 1f and g), suggesting that the mechanical tension and growth factors present in the granulation tissue of SM α A-null mice are sufficient to activate the SM α A promoter.

SM α A-null fibroblasts can acquire a myofibroblast phenotype

Myofibroblasts are characterized by the formation of stress fibers and supermature focal adhesions, which are focal adhesions above $6\ \mu\text{m}^2$ (3, 5, 6). WT dermal fibroblasts, cultured for 4 days with TGF- β 1 to promote myofibroblast formation, immunostained with clone 1A4, which recognizes SM α A, while SM α A-null dermal myofibroblasts did not stain confirming the absence of SM α A in these cells. (Figure 2a). Both WT and SM α A-null fibroblasts, cultured for 4 days with TGF- β 1, assembled large stress fibers, visualized by phalloidin staining, and supermature focal adhesions, visualized by staining with anti-vinculin antibody (Figure 2b). Focal adhesion size was quantified by image analysis of WT and SM α A-null fibroblasts and myofibroblasts. Supermature focal adhesions formed to the same extent in SM α A-null and WT fibroblasts, and increased in both cell types in response to TGF- β 1 (Figure 2c). These results demonstrate that SM α A-null fibroblasts can acquire the myofibroblast phenotype in response to TGF- β 1 and that expression of SM α A is not necessary for the formation of large stress fibers and supermature focal adhesions.

SM α A-null fibroblasts can generate increased contractile force in response to TGF- β 1

Next, we examined whether SM α A-null fibroblasts can generate contractile force that increases with TGF- β 1 treatment. Two methods were used to evaluate contractile force generation. The first method used was the stressed-relaxed collagen lattice contraction assay (3, 11, 19). WT and SM α A-null fibroblasts were cultured within a stressed three-dimensional collagen lattice for 5 days without or with TGF- β 1, after which the lattice was released and evaluated for changes in collagen lattice diameter. Non-treated WT and SM α A-null fibroblasts contracted collagen lattices similarly (Figure 3a). Treatment with TGF- β 1 increased collagen lattice contraction of WT and SM α A-null fibroblasts to a similar extent (Figure 3a). The second method used the culture force monitor which directly measures the level of force transmitted by fibroblasts to collagen fibers in real time (31). WT and SM α A-null fibroblasts placed within a three-dimensional collagen lattice and attached to the culture force monitor produced the characteristic force generation curve observed for all types of fibroblasts (Figures 3b and c). Both the increase in force observed

over the first 6 hours and the maximum force plateau reached was similar in WT and SM α A-null fibroblasts. To promote myofibroblast differentiation, WT and SM α A-null fibroblasts were treated for 5 days with TGF- β 1 prior to placement in the culture force monitor. WT and SM α A-null myofibroblasts produced identical force generation curves and maximum force plateaus; in addition, WT and SM α A-null myofibroblasts generated greater force than did the fibroblasts (Figures 3b and c). Together these results demonstrate that lack of SM α A does not reduce contractile force generation.

Other muscle actin isoforms are expressed by myofibroblasts that may compensate for loss of SM α A expression

The above results demonstrate that SM α A expression is not necessary for myofibroblast formation and function. To determine whether other muscle actin isoforms are expressed in myofibroblasts, SM α A-null granulation tissue was immunostained with clone B4, which recognizes all muscle actin isoforms (Table 1). As expected, the staining pattern of clone B4 in WT granulation tissue (Figure 4a) was similar to that observed when stained with clone 1A4, which recognizes SM α A (not illustrated). Clone B4 stained SM α A-null granulation tissue in a pattern similar to WT granulation tissue, demonstrating the expression of other muscle actin isoforms in the absence of SM α A (Figure 4b). Clone B4 also stained bundles of actin microfilaments that appeared comparable to stress fibers in both WT and SM α A-null myofibroblasts in granulation tissue (Figures 4c and d). Vascular smooth muscle in SM α A-null mice stained with the clone B4 (Figure 4b), demonstrating that these cells also express other muscle actin isoforms in the absence of SM α A. These results demonstrate that fibroblasts in SM α A-null granulation tissue express other muscle actin isoforms comparable to the expression of SM α A in myofibroblasts in WT granulation tissue and organize these other muscle actin isoforms into stress fibers, demonstrating that SM α A-null fibroblasts in granulation tissue can acquire the myofibroblast phenotype.

We examined whether knockout of SM α A altered levels of total actin, CY β A, or CY γ A. No differences in the level of total actin was observed by Western blotting with clone C4 between WT and SM α A-null fibroblasts cultured with TGF- β 1 for 4 days to promote myofibroblast formation (Figure 5a). Similarly, Western blotting for either CY β A (clone 4C2) or CY γ A (clone 2A3) showed no increase in SM α A-null compared with WT myofibroblasts (not illustrated). In addition, there was no significant change in mRNA for either CY β A or CY γ A between WT and SM α A-null myofibroblasts (Figure 6). Lack of SM α A expression in SM α A myofibroblasts was verified by Western blotting with clone 1A4 (Figure 5a), real time RT-PCR for SM α A mRNA (Figure 6a and b), and mass spectrometry (Supplemental Figure S3).

Next, we examined whether other muscle actin isoforms are expressed by cultured dermal fibroblasts after treatment with TGF- β 1 to promote myofibroblast formation. Western blotting with clone B4 gave similar actin bands in both WT and SM α A-null myofibroblasts (Figure 5b). In addition, both WT and SM α A-null myofibroblasts stained with clone 4B in a stress fiber pattern (Figure 7a), and a similar percentage of WT and SM α A-null myofibroblasts stained with the clone B4 (53.7% WT; 45.6% SM α A-null). These results demonstrate that cultured SM α A-null myofibroblasts express other muscle actin isoforms, similar to our observations in SM α A-null granulation tissue, and that a similar percent of WT and SM α A-null myofibroblasts stain for all muscle actin isoforms.

To determine whether SM γ A is expressed in SM α A-null myofibroblasts, we used clone CGA7, which recognizes SM γ A and SM α A. Clone CGA7 immunostained both WT and SM α A-null myofibroblasts in a stress fiber pattern (Figures 7c and d). Significantly fewer of the SM α A-null myofibroblasts (21.5%) immunostained with clone CGA7 than WT myofibroblasts (55%) ($p = 0.001$); however, clone CGA7 immunostains SM α A and SM γ A

present in WT myofibroblasts and only SM γ A in SM α A-null myofibroblasts. Western blotting with clone CGA7 demonstrated a band at the molecular weight for actin in WT myofibroblasts but only a very faint band in SM α A-null myofibroblasts (Figure 5b), suggesting that the level of SM γ A in SM α A-null myofibroblasts is significantly less than the level of SM α A in WT myofibroblasts. Consistent with these results, mRNA for SM γ A is significantly increased in SM α A-null myofibroblasts compared with WT myofibroblasts demonstrating increased expression of SM γ A with loss of SM α A; however, the level of mRNA for SM γ A in SM α A-null myofibroblasts is approximately 5-times lower than the level of mRNA for SM α A in WT myofibroblasts (Figure 6b). Mass spectrometry confirmed the presence of SM γ A in both WT and SM α A-null myofibroblasts (Supplemental Figure S3). Together these results demonstrate that SM γ A is expressed in SM α A-null myofibroblasts; however, the level of expression appears to be significantly less than that of SM α A in WT myofibroblasts.

To determine whether SkM α A and CM α A are expressed in SM α A-null myofibroblasts, we used clone 5C5. Western blotting with clone 5C5 did not give an actin band for either cell type (Figure 5b), although it did recognize an actin band in mouse skeletal muscle used as a positive control (not illustrated). Clone 5C5 immunostained SM α A-null and WT myofibroblasts in a stress fiber pattern (Figure 7c) and significantly more SM α A-null myofibroblasts stained than WT myofibroblasts (17.1% WT; 35.7% SM α A-null; $p < 0.05$). Mass spectrometry confirmed the presence of a peptide shared by both SkM α A and CM α A in SM α A-null and WT myofibroblasts (Supplemental Figure 3). SM α A-null fibroblasts and myofibroblasts did express significantly higher mRNA for SkM α A than their WT counterparts (Figure 7); however, the level of mRNA for SkM α A in SM α A-null myofibroblasts is approximately 4-times lower than the level of mRNA for SM α A in WT myofibroblasts (Figure 6b). No mRNA for CM α A was observed (not illustrated), suggesting myofibroblasts are not expressing CM α A. Taken together these results demonstrate that SM α A-null myofibroblasts express SkM α A at higher levels than WT myofibroblasts; however, the level of expression appears to be less than the level of expression of SM α A in WT myofibroblasts.

DISCUSSION

In this paper we demonstrate that mice lacking SM α A can close excisional wounds and that the mechanical and growth factor environment present in the granulation tissue of these SM α A-null mice is sufficient to promote activation of the SM α A promoter. In addition, we demonstrate that mouse dermal fibroblasts lacking SM α A can form stress fibers and focal adhesions and can generate increased contractile force in response to TGF- β 1, similar to WT fibroblasts. There was a significant delay early in the closure of 4 mm wounds in SM α A-null mice; however, this delay disappeared by 4 days after wounding. It is unclear why lack of SM α A expression should delay early wound closure, as SM α A-positive myofibroblasts appear later during wound closure (3, 33). Previous studies have demonstrated a correlation between SM α A-positive myofibroblasts and tissue contraction, suggesting a role for SM α A in generating cellular contractile force (3, 8, 34). In addition, increased expression of SM α A will result in increased formation of stress fibers and focal adhesions and in increased contractile force generation (3, 11). Therefore, at first glance it is surprising that SM α A-null wounds closed and SM α A-null fibroblasts could acquire the myofibroblast contractile phenotype and force generation; however, a number of studies on other cell types have demonstrated that other muscle actin isoforms can, at least partially, compensate for the lack of a muscle actin isoform (15, 16, 35, 36). These results would suggest that myofibroblasts have a mechanism for compensating for the lack of expression of SM α A, perhaps by expression of other muscle actin isoforms.

Fibroblasts in SM α A-null granulation tissue were found to express muscle actin isoforms other than SM α A, as evidenced by immunostaining with clone B4, and organize these muscle actin isoforms into stress fiber-like structures, suggesting that other muscle actin isoforms are expressed and can potentially compensate for the lack of SM α A. Cultured SM α A-null mouse dermal fibroblasts treated with TGF- β 1 to promote myofibroblast formation were found to express both SM γ A and SkM α A. The loss of SM α A in myofibroblasts resulted in no decrease in total actin levels and no increase in CY β A or CY γ A, demonstrating that the expression of these two cytoplasmic actin isoforms did not increase to compensate for the loss of SM α A in myofibroblasts. SM γ A was expressed in SM α A-null myofibroblasts as demonstrated by immunostaining with clone CGA7 and real time RT-PCR. Because clone CGA7 recognizes SM α A, in addition to SM γ A, it was not possible to determine whether SM γ A protein is increased in SM α A-null compared with WT myofibroblasts; however, SM γ A mRNA is significantly increased in SM α A-null compared with WT myofibroblasts, suggesting that SM γ A is increased in response to lack of SM α A. SkM α A was observed to increase in SM α A-null compared to WT myofibroblasts both by immunostaining with clone 5C5 and by RT-PCR. The level of expression of SM γ A and SkM α A in SM α A-null myofibroblasts appears to be significantly less than expression of SM α A in WT myofibroblasts; whether the expression of SM γ A and SkM α A occurs in the same cell, reaching the level of SM α A expression, is unclear. While we have demonstrated that SM γ A and SkM α A are expressed and organized into stress fibers in SM α A-null myofibroblasts, further studies are necessary to determine the relative level of expression of these actin isoforms to each other and to the expression of SM α A in WT myofibroblasts.

The mechanism by which SM γ A and SkM α A may functionally compensate for the loss of SM α A is currently unknown. Consistent with our findings, Chambers and co-workers have demonstrated that human fetal lung fibroblasts treated with TGF- β 1 increase SM α A, SM γ A, and CM α A expression (12). We did not see expression of CM α A in myofibroblasts, suggesting that myofibroblasts from different locations may express different muscle actin isoforms in response to TGF- β 1. The amino acid sequences for the six actin isoforms are conserved for vertebrates, suggesting strong evolutionary pressure to maintain these specific sequences and differential function for each isoform (9). Currently the function for the different actin isoforms is not completely understood. Studies have suggested that in non-muscle cells the two cytoplasmic actin isoforms, CY β A and CY γ A, have different localizations and different functions (5, 37, 38). Several lines of evidence suggest that other muscle actin isoforms can functionally compensate, at least partially, in CM α A-null mice (35) and SkM α A-null mice (36). Similarly, SM α A-null mice have no major phenotype and SkM α A has been proposed to functionally compensate, at least partially, in vascular smooth muscle cells (15). Myoepithelial cell function is dramatically decreased in SM α A-null mice such that lactating females cannot nurse their pups (17, 18). This inability for compensation may be due to lack of expression of other muscle actin isoforms in these epithelial-derived contractile cells. Interestingly, human genetic disorders have recently been described with mutations in SM α A which cause cardiovascular defects; in this case, compensation may not be possible since these mutations appear to be dominant negative (39). Myofibroblast contractile function has been demonstrated to be perturbed by the peptide, Ac-EEED, homologous to the N-terminal sequence of SM α A (33). These results suggest that the acute perturbation of SM α A function may not allow time for compensation mechanisms to come into play, as can occur in null animals. Consistent with this, acute knockdown of SM α A using antisense oligonucleotides decreased focal adhesion formation and increased cell migration (40). Recent work has demonstrated that there is a broad genetic reprogramming of β -actin-null cells that does not occur in acute knockdown of β -actin (38). It may be that whole animal knockout of SM α A results in a genetic reprogramming that allows for compensation that does not occur with acute knockdown or loss of function of SM α A. Further experiments comparing SM α A-null with acute SM α A knockdown in fibroblasts

will be needed to determine the mechanisms for compensation and possible genetic reprogramming.

In cardiac and smooth muscle cells, expression of muscle actin isoforms and other contractile proteins is regulated by the transcription factor, myocardin, through its binding to serum response factor and to CArG elements in the promoter of these genes (41). Non-muscle cells do not express myocardin; rather, they express the related transcription factors myocardin-related transcription factor A and B (MRTF-A, MRTF-B) (42). We have recently demonstrated that knockdown of MRTF-A and -B in myofibroblasts will significantly reduce expression of SM α A and SM γ A and other contractile proteins, and significantly reduce the myofibroblast contractile phenotype and its ability to generate contractile force (13). These results are consistent with the results presented in the current study, in that loss of expression of SM α A can be compensated for, and that knockdown of all muscle actin isoforms and other contractile proteins is required to reduce myofibroblast formation and function.

TGF- β 1 treatment of SM α A-null fibroblasts significantly increases SkM α A mRNA, increases SM γ A mRNA, although not significantly, and increases significantly mRNA for SM22 α and h1-calponin (Tomasek, unpublished results), all of which may play a role in promoting myofibroblast formation and function in the absence of SM α A. The mechanism by which TGF- β 1 may promote increased expression of SkM α A and SM α A is currently unclear. We have previously proposed that TGF- β 1 may promote expression of myofibroblast contractile genes by directly altering actin dynamics through a Rho-mediated mechanism and thereby promote nuclear translocation of MRTF-A/B (13). SM γ A and SK α A promoters have CArG elements that bind SRF that may be regulated by MRTF-A/B (43–45). However, TGF- β 1 has also been shown to increase expression of SM α A and SM22 α in fibroblasts through TGF- β 1 control elements (46–48) and/or Smad-binding elements (49, 50). Further studies will be needed to understand how TGF- β 1 promotes increased expression of these contractile proteins and their role in promoting myofibroblast formation and function in SM α A-null fibroblasts.

We have demonstrated that loss of SM α A in myofibroblasts results in increased expression of SM γ A and SkM α A, and that the expression of these muscle actin isoforms may functionally compensate for the loss of SM α A. Myofibroblasts express, in addition to muscle actin isoforms, other contractile proteins such as SM22 α and h1-calponin, which also appear to regulate myofibroblast formation and function. To understand myofibroblast formation and function it will be necessary to identify the multiple contractile proteins expressed, to understand how their expression is regulated, and to determine how they interact to promote the contractile phenotype and force generation so important in wound closure and pathological contractures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

SMαA expression is not necessary for closure of excisional wounds in mice. **a:** Macroscopic appearance of WT and SMαA-null (SMαA^{-/-}) mice with 7 mm diameter wounds at indicated time points after wounding; no differences were observed. **b and c:** Excisional wounds of 4 mm (**b**) or 7 mm (**c**) were made on WT and SMαA-null mice. Wound area was determined using image analysis and expressed as the percentage of wound closure. n=6. **p*<0.05. **d and e:** Hematoxylin and eosin stained sections of 12 day granulation tissue from WT (**d**) and SMαA-null (**e**) mice illustrating granulation tissue and cut edges of wound; bar=300 μm. **f and g:** Sections of 12 day granulation tissue from SMαA -null mice carrying the p2600Int/LacZ transgene stained with hematoxylin and eosin and for β-galactosidase (**f**) or β-galactosidase only (**g**); bar=200 μm.

Figure 2.

SMαA-null fibroblasts can acquire the myofibroblast contractile phenotype. **a:** WT and SMαA-null (SMαA^{-/-}) fibroblasts were cultured for 4 days in the presence of TGF-β1, immunostained with anti-SMαA antibody, and stained with DAPI to detect nuclei. Stress fibers in WT fibroblasts are stained for SMαA, while no immunostaining is observed in SMαA-null fibroblasts. Bar=100 μm. **b:** WT and SMαA-null (SMαA^{-/-}) fibroblasts were cultured for 4 days in the presence or absence of TGF-β1. Stress fibers were visualized with phalloidin and focal adhesions with anti-vinculin antibody. No differences between WT and SMαA-null fibroblasts were observed. Bar=150 μm. **c:** WT and SMαA-null fibroblasts were cultured for 4 days in the absence (WT fibroblasts, Null fibroblasts) or presence (WT myfibroblasts, Null myfibroblasts) of TGF-β1. Cells were immunostained with anti-vinculin antibody, and focal adhesion size quantified. Focal adhesion size increased similarly in response to TGF-β1 in WT and SMαA-null fibroblasts.

Figure 3.

SMαA-null fibroblasts increase generation of contractile force in response to TGF-β1 similar to WT fibroblasts. **a**: Stress-relaxed collagen lattices were used to determine contractile force generation. WT and SMαA-null fibroblasts contracted lattices similarly and both cell types increased contraction similarly in response to TGF-β1. All contraction assays were carried out in triplicate, and every experiment was repeated three or more times. **b–c**: Contractile force generation was investigated using a culture force monitor. No difference was found in force (dynes) generation by WT (b) or SMαA-null (c) fibroblasts and both cell types increased force similarly in response to TGF-β1. Representative traces are shown from three different experiments.

Figure 4.

SM α A-null fibroblasts in 12 day granulation tissue express muscle actin isoforms other than SM α A and organize this muscle actin into stress fiber-like structures. Cryosections of 12 day granulation tissue from WT (a, c) and SM α A-null (b, d) mice were immunostained with clone B4 which recognizes all muscle actin isoforms. Clone B4 immunostains SM α A-null (b) and WT (a) granulation tissue in a similar pattern. Stress fiber-like structures were immunostained in myofibroblasts in WT (c) and SM α A-null granulation tissue (d). Vascular smooth muscle cells were immunostained in both WT and SM α A-null granulation tissue. a–d: bar=50 μ m.

Figure 5.

Identification of actin isoforms expressed in WT and SMαA-null myofibroblasts by Western blotting. WT and SMαA-null fibroblasts were cultured with TGF-β1 for 4 days to promote myofibroblast formation. Cells were then lysed, and equal levels of protein from each sample loaded on the gel. **a:** Western blotting was performed using clone C4 to determine total actin levels and clone 1A4 to determine SMαA levels. **b:** Western blotting was performed using clone B4 to determine level of all muscle actin isoforms, clone CGA7 to determine SMγA and SMαA levels, and clone 5C5 to determine SkMαA and CMαA levels. Arrow indicates molecular weight of actin. Molecular weight marker sizes are indicated.

Figure 6. Identification of actin isoforms expressed in WT and SMαA -null fibroblasts and myofibroblasts by real time RT-PCR. WT and SMαA-null fibroblast were cultured with TGF-β1 for 4 days, and expression of mRNA for actin isoforms was examined by real-time RT-PCR. **a:** mRNA levels were normalized to WT control for each actin isoform to evaluate increase of specific actin isoform in response to treatment. **b:** mRNA levels were normalized to b-actin mRNA level in WT myofibroblasts to compare relative levels of mRNA of different actin isoforms. n=4 dishes/treatment.

Figure 7.

Cultured SM α A-null myofibroblasts immunostain for all muscle actin isoforms, SM γ A, and SkM α A, and organize these actin isoforms into stress fibers. WT (**a, c, e**) and SM α A-null (**b, d, f**) fibroblasts were cultured with TGF- β 1 for 4 days and immunostained with clone B4 monoclonal antibody, which recognizes all muscle actin isoforms (**a, b**); clone CGA7 monoclonal antibody, which recognizes SM α A and SM γ A actin isoforms (**c, d**); or clone 5C5 monoclonal antibody, which recognizes SkM α A and CM α A actin isoforms (**e, f**). bar=50 μ m.

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Table 1
A description of actin isoforms recognized by monoclonal antibodies used in this study

	Clone C4	Clone 4C2	Clone 2A3	Clone B4	Clone 1A4	Clone CGA7	Clone 5C5
CYβ-A	Yes	Yes	No	No	No	No	No
CYγ-A	Yes	No	Yes	No	No	No	No
SMαA	Yes	No	No	Yes	Yes	Yes	No
SMγA	Yes	No	No	Yes	No	Yes	No
SKMαA	Yes	No	No	Yes	No	No	Yes
CMαA	Yes	No	No	Yes	No	No	Yes