Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. Mouse NIH/3T3 and human BR5 fibroblasts were grown as in DMEM supplemented with 10% BCS or FBS at 37 °C in a 5% CO_2 incubator as previously described (1). Primary mouse tail tip fibroblasts (TTFs) were isolated from 6- to 8-wk-old myocardin-related transcription factor (MRTF)-A$^{-/-}$ or wild-type littermates and cultured in DMEM supplemented with 10% FBS, nonessential amino acids, L-glutamine, and sodium pyruvate as described previously (2). Once TTFs became confluent, cells were frozen in 10% DMSO containing growth media for future use before passage 3.

NIH/3T3 cells were transfected with 10 ng empty flag or flag epitope-tagged MRTF-A vector along with 100 ng smooth muscle α-actin (SMA), atrial natriuretic factor (ANF), SM22, or skeletal actin (SKA) luciferase reporter and 20 ng of β-galactosidase (β-gal) expression construct using Fugene6 (Roche). Plasmid DNA content was kept constant using empty pcDNA3.1-flag. BR5, NIH/3T3, or TTF cells were infected with adenovirus directing the expression of flag–MRTF-A, HA–MRTF-A, or β-gal as control at the multiplicity of infection (MOI) noted in the text. For experiments using chemical compounds or growth factors, cells were cultured in serum-free media (SF) for 24 h before treatment, followed by an additional 24- to 48-h incubation in SF media treated with TGF-β1 (10 ng/mL, R&D Systems) solubilized in 4 mM HCl/1% BSA/PBS or isoxazole (20 μM) solubilized in DMSO.

Immunocytochemistry. Cells were treated with the indicated factor for 24 h before fixation with methanol at −20 °C for 10 min. Indirect immunofluorescence was performed by incubating with the following primary antibodies at 4 °C overnight: mouse monoclonal Cy3-conjugated anti-SMA antibody (Sigma, clone 1A4, 1:200) and rabbit monoclonal anti-HA antibody (Cell Signaling, 1:300). A secondary goat Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Invitrogen, 1:200) was used to visualize HA by incubation at RT for 1 h. All images were captured using an Olympus IX81 confocal microscope.

Collagen Gel Contraction Assay. BR5 cells (2×10^5) were infected with 25 MOI flag-MRTF-A, or GFP as control, for 48 h and seeded in a 200 μL volume of 1.5 mg/mL rat tail collagen (BD Bioscience), essentially as described (1). The BR5-containing collagen matrix was dispensed onto 12 mm diameter circles scored on the bottom of a 24-well tissue culture plate. Gels were allowed to polymerize for 1 h. Gels for floating matrix contraction were then released from the tissue culture plastic followed by an overnight incubation in DMEM supplemented with 10% FBS to allow tension development. Gels for stressed matrix contraction were released from the tissue culture plastic after overnight in 10% FBS. Reduction of gel diameter in response to BSA control (5 mg/mL), FBS (10%), or PDGF (50 ng/mL) was calculated.

Western Blot Analysis. Whole cell extracts were subjected to SDS/PAGE and immunoblotted onto PVDF membranes (Millipore) with the following antibodies at 4 °C overnight: mouse monoclonal anti-SMA (Sigma, clone 1A4, 1:1,000), rabbit polyclonal anti-SM22 (Abcam, 1:5,000), rabbit monoclonal Smad2/3 and phoshpho-Smad2/3 (Cell Signaling, 1:1,000), rabbit monoclonal Erk1/2 and phsopho Erk1/2 (Cell Signaling, 1:1,000), SRF (Santa Cruz, 1:1,000), rabbit monoclonal Vimentin (Abcam, 1:1,000), rabbit polyclonal Fsp1 (S100a4, 1:1,000), mouse monoclonal anti-FlagM2 (Sigma, 1:5,000), and mouse monoclonal anti-GAPDH (Millipore, 1:30,000). Secondary goat HRP-conjugated anti-mouse or –rabbit IgG antibody (BioRad) was incubated for 1 h at room temperature and developed with luminol reagent (Santa Cruz).

RNA Isolation and Analysis. Total RNA was isolated from cell cultures and tissue samples using TRIzol reagent (Invitrogen), and cDNA was generated using iScript cDNA Synthesis Kit (BioRad) following the manufacturer's protocol. Gene expression was examined using standard RT-PCR methods and visualized by gel electrophoresis or qRT-PCR methods with iQ SYBR Green Supermix (BioRad). Primer sequences, annealing temperature, and cycle numbers are listed in Table S1.

Mouse Lines and Wound-Healing Assay. All experiments using animals were previously approved by the University Committee on Animal Resources at University of Rochester and the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. The MRTF-A$^{-/-}$ mouse line used in this study has been previously reported and is kept on a C57Bl6 background (3). Wild-type C57BL6 mice were obtained from the Jackson Laboratories. Wound-healing assays were performed as previously reported (4). Briefly, mice were shaved down the back and wiped with betadine followed by full-thickness incision with a biopsy punch (4 mm). Two biopsies were performed per mouse, and care was taken to assure equal skin tension for all wounds. Isoxazole (ISX) (20 μM in PBS) or an equal concentration of DMSO was applied to each mouse twice per day in an ~50-μL volume for 7 d. At the terminus of the study, wounds and control skin were excised and frozen in liquid nitrogen for RNA and protein isolation or fixed for histology.

Histology. All samples were fixed in 4% paraformaldehyde, processed, paraffin-embedded, sectioned at 5-μm thickness, and stained for H&E or Masson's trichrome. For SMA immunofluorescence, sections were incubated with anti-SMA-Cy3 antibody (1:200) at 4 °C overnight. For Ki-67 detection, sections were pressure-cooked in antigen retrieval buffer (DAKO, pH 6.0) for 20 min and then incubated with rat anti-KI-67 antibody (DAKO, clone TEC-3, 1:500) at 4 °C overnight. Sections were then incubated with secondary biotinylated rabbit anti-rat IgG antibody (DAKO, 1:200) and ABC complex (Vector), each for 30 min at room temperature and developed with diaminobenzidine peroxidase substrate (Vector) for 10 min. Wounds were imaged using a dissecting scope or an Olympus IX81 confocal microscope and analyzed with National Institutes of Health ImageJ.

1. Rhee S, Jiang H, Ho CH, Grinnell F (2007) Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions. *Proc Natl Acad Sci USA* 104(13):5425–5430.
2. Song K, et al. (2012) Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 485(7400):599–604.
3. Li S, Chang S, Qi X, Richardson JA, Olson EN (2006) Requirement of a myocardin-related transcription factor for development of mammary myoepithelial cells. *Mol Cell Biol* 26(15):5797–5808.
4. Dioufa N, et al. (2010) Acceleration of wound healing by growth hormone-releasing hormone and its agonists. *Proc Natl Acad Sci USA* 107(43):18611–18615.
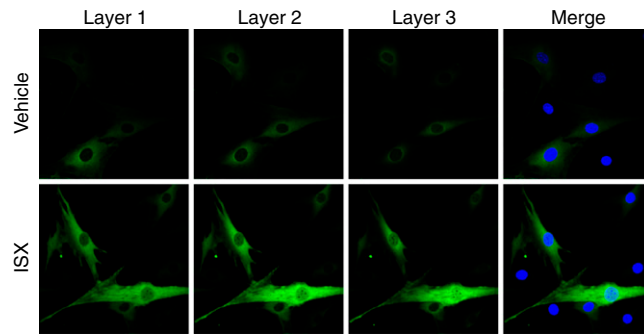
Fig. S1. ISX promotes MRTF-A enrichment and nuclear accumulation. BR5 human dermal fibroblasts were infected with HA–MRTF-A and treated with vehicle or ISX for 24 h. Immunofluorescence for HA (green) or DAPI (blue, nuclei) demonstrates MRTF-A accumulation and nuclear localization. Selected images from various levels taken from a confocal Z-stack at lower (layer 1), midcell (layer 2), and upper (layer 3).
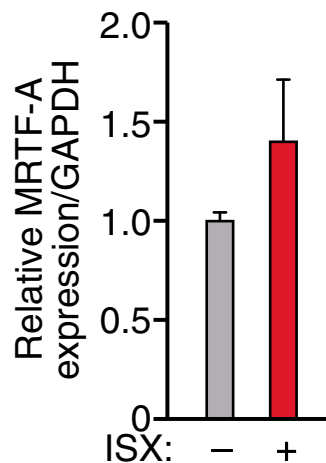
Fig. S2. MRTF-A mRNA levels are unchanged by ISX. NIH3T3 cells infected with HA–MRTF-A (25 MOI) and treated with ISX or vehicle. MRTF-A mRNA levels were analyzed using qRT-PCR and normalized to GAPDH.

Fig. S3. Effect of ISX on wound healing. Wild-type mice were subjected to two wounds; one wound was treated with 20 μM ISX twice per day and the second with DMSO (vehicle) as an internal control. Wound was imaged 7 d following punch biopsy.

Fig. S4. Inflammatory marker analysis in ISX-treated wounds. Biopsies were isolated from wounds treated with ISX (20 μM) or vehicle, two times per day for 7 d. qRT-PCR analysis of inflammatory markers ($n = 4$, **$P < 0.01$).

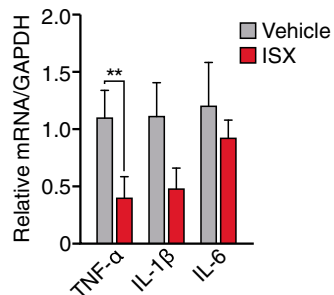**Fig. S5.** Proliferation is not affected by ISX treatment of wounds. Number of Ki67-positive nuclei within the granulation tissue per field of view in ISX- or vehicle-treated wounds after 7 d.

Fig. S6. Cutaneous wound healing in MRTF-A–null mice. (A) Wild-type or MRTF-A KO mice were subjected to full-thickness cutaneous wounds and imaged over the course of 11 d. (B) Quantification of the wound areas using National Institutes of Health ImageJ (n = 10). (C) H&E (a, d) and Masson's trichrome staining (b, c, e, f) of WT or MRTF-A KO wounds depicting the migrating epidermal sheets (black arrow) and granulation tissue area [4× (a, b, d, e) and 40× (c, f) magnification]. [Scale bar, 500 μm (4×) or 50 μm (40×).]

Fig. S7. *SRF* and *MRTF-B* expression in wounds from WT and MRTF-A KO mice. Gene expression was examined by RT-PCR from biopsies taken from full-thickness cutaneous wounds or control unwounded skin, 7 d after injury in mice. Expression levels were examined for three independent mice per genotype, using GAPDH as loading control. (−) denotes control unwounded skin; (+) denotes wound tissue.

Table S1. Primers used in this study

Gene	Forward	Reverse
mus COL1A1	TAG GCC ATT GTG TAT GCA GC	ACA TGT TCA GCT TTG TGG ACC
mus COL1A2	AGC AGG TCC TTG GAA ACC TT	AAG GAG TTT CAT CTG GCC CT
mus COL3A1	TAG GAC TGA CCA AGG TGG CT	GGA ACC TGG TTT CTT CTC ACC
mus GAPDH	CGT GCC GCC TGG AGA AAC	TGG GAG TTG CTG TTG AAG TCG
mus IL-1β	CTA CAG GCT CCG AGA TGA AC	TTC TTC TTT GGG TAT TGC TTG G
mus IL6	ACA AAG AAA TGA TGG ATG CTA CC	GTA TCT CTC TGA AGG ACT CTG G
mus MRTF-A	ACG AGG CGG TTA CCA TCA C	GCA GAC AGA GAC AGG AGC AC
mus MRTF-B	CGA TAG CTC CAA GAA GCA GC	TTT TCT GGT TGC TTC CCT CA
mus MYOCD	AAG GTC CAT TCC AAC TGC TC	CCA TCT CTA CTG CTG TCA TCC
mus SM22	GAC TGC ACT TCT CGG CTC AT	CCG AAG CTA CTC TCC TTC CA
mus SMA	GTT CAG TGG TGC CTC TGT CA	ACT GGG ACG ACA TGG AAA AG
mus SRF	CAC CTA CCA GGT GTC GGA AT	GTC TGG ATT GTG GAG GTG GT
mus TNF-α	TCT TCT CAT TCC TGC TTG TG	ACT GGG TGG TTT GCT ACG

PCR parameters: denature 95 °C, 30 s; annealing 60 °C, 30 s; extension 72 °C, 30 s; 30× cycles.

Table S2. Antibodies used for Western blots in this study

Antigen	Source	Host	Dilution
ERK1/2	Cell Signaling	Rabbit	1:5,000
Phospho Erk1/2	Cell Signaling	Rabbit	1:5,000
FlagM2	Sigma	Mouse	1:5,000
GAPDH	Millipore	Mouse	1:30,000
S100a4	Abcam	Rabbit	1:500
SMA	Sigma, clone 1A4	Mouse	1:1,000
SMAD2/3	Cell Signaling	Rabbit	1:1,000
Phospho-Smad2/3	Cell Signaling	Mouse	1:1,000
SRF	Santa Cruz	Rabbit	1:1,000
Vimentin	Abcam	Rabbit	1:5,000