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Mesenchymal stem cells have anti-fibrotic effects on Transforming Growth Factor- β 1-stimulated vocal fold fibroblasts

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Abstract

Objectives/Hypothesis—Mesenchymal stem cells (MSCs) hold therapeutic promise for vocal fold scar, yet the precise mechanism(s) underlying tissue level changes remain unclear. We hypothesize that MSCs interact with native fibroblasts to favorably affect healing. Furthermore, we hypothesize these interactions vary based on MSC source.

Methods—Vocal fold fibroblasts (VFFs), adipose-derived stem cells (ASCs), and bone-marrow derived stem cells (BMSCs) were extracted from Sprague-Dawley rats and a co-culture model was employed culturing VFFs+/-TGF- β 1 (10ng/ml) +/- MSCs. Mono-culture MSCs were also prepared as a control. Both extracellular matrix (ECM) and components of the TGF- β signaling pathway were analyzed via polymerase chain reaction and SDS-PAGE.

Results—Significantly decreased TGF- β 1 mRNA and α -smooth muscle actin protein was observed in VFFs in response to TGF- β 1 in the co-culture with both MSCs ($p < 0.05$, $p < 0.01$). BMSCs significantly downregulated Collagen I ($p < 0.05$), Collagen III ($p < 0.05$), Smad3 ($p < 0.01$), and TGF- β 1 receptor I ($p < 0.01$) mRNA in VFFs. Hyaluronic synthase-1 and 2 increased in co-cultured BMSCs when compared with mono-cultured BMSCs at baseline and in response to TGF- β 1 ($p < 0.01$).

Conclusions—MSCs had a favorable effect on ECM regulation as well as suppression of TGF- β 1 signaling in VFF. Bidirectional paracrine signaling was also observed as VFFs altered ECM regulation in MSCs. These data provide insight into the regenerative effects of MSCs and provide a foundation for clinical application.

Keywords

vocal fold; voice; fibrosis; mesenchymal stem cells; extracellular matrix; TGF- β

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INTRODUCTION

Vocal fold (VF) fibrosis can result from various injuries including phonotrauma, inflammation, or surgery. Given that optimal tissue pliability and viscoelasticity are required for normal voice, VF fibrosis can yield substantive dysphonia due to altered biomechanical properties. Changes to the extracellular matrix (ECM) associated with VF fibrosis include excessive collagen deposition, disorganized elastin, and loss of hyaluronic acid (HA).¹⁻³ These tissue changes pose a significant clinical challenge as current therapeutics remain suboptimal. Stem cell therapy has evolved in the field of regenerative medicine. Mesenchymal stem cells (MSCs) are considered a promising therapeutic tool and clinical trials are currently underway for a variety of diseases including myocardial ischemia, graft-versus-host diseases, liver cirrhosis, anemia, Alzheimer's disease, spinal cord injury, and ischemic stroke.⁴ The main mechanism underlying the therapeutic effect of MSCs is likely paracrine effects via tropic factors with anti-fibrotic, anti-apoptotic, and/or pro-angiogenic properties.⁵

MSCs are safe and pose no ethical issues, particularly when employing an autologous source. Furthermore, isolation of MSCs is not challenging and can be obtained from a wide range of tissues. The regenerative efficacy of MSCs for vocal fold fibrosis has been previously reported in a variety of species;⁶⁻¹⁰ however, several critical foundational issues must be addressed including cell source, cell quantity, and timing of delivery. A recent study described injection of adipose-derived stem cells (ASCs) and bone marrow-derived stem cells (BMSCs) into the injured rat VF submucosa. No differences were reported between cell type with regard to healing outcomes.¹¹ Insight into the utility of MSCs as a therapeutic tool for the management of vocal fold injury and repair is limited and warrants further investigation.

Wound healing involves a complex network of cells and extracellular components. Fibroblasts and myofibroblasts synthesize ECM and reconstruct the microenvironment following injury.¹² Mature myofibroblasts express alpha smooth muscle actin (α -SMA) and produce excessive collagen yielding increased tissue stiffness. Persistent myofibroblast activation and collagen deposition underlies a variety of fibrotic processes.¹³ Transforming growth factor (TGF)- β is a central mediator of myofibroblast differentiation and increased TGF- β mRNA expression has been reported as early as eight hours following VF injury.¹⁴ Furthermore, *in vitro*, TGF- β has been shown to regulate the fibrotic phenotype in vocal fold fibroblasts; this response appears to be Smad3-dependent.¹⁵ Conversely, hepatocyte growth factor (HGF) has been shown to be anti-fibrotic. Exogenous HGF enhanced histological and functional healing in both acute and more chronic vocal fold fibrosis in canine mode.^{16,17} In the current study, we hypothesized that MSCs regulate TGF- β signaling, HGF secretion, and ECM production in VF fibroblasts via paracrine signaling. We employed a co-culture model to address this hypothesis. In addition, we hypothesized these paracrine effects vary based on the source of MSCs (ASCs vs BMSCs). Finally, we hypothesized that this paracrine signaling is likely bidirectional with VFFs affecting the MSC activity. Ultimately, exploiting the inherent cellular response following vocal fold injury to direct the wound healing toward a more regenerative outcome, holds significant therapeutic promise.

MATERIALS AND METHODS

Isolation and confirmation of primary culture

Following approval from the Institutional Animal Use and Care Committee at the New York University School of Medicine, Sprague-Dawley (SD) rats (5 females aged between 12 to 14 weeks) were employed to create primary culture of three cell types; vocal fold fibroblasts (VFFs), adipose-derived stem cells (ASCs), and bone marrow-derived stem cells (BMSCs). Isolation of VFFs was performed according to previously-published methods.^{18,19} Briefly, the lamina propria (LP) and epithelia of the VFs were resected and minced under magnification. Isolation of MSCs was described previously.¹¹ ASCs were isolated from minced inguinal fat pads with Liberase (Roche, Indianapolis, IN) according to the manufacturer's protocol. BMSCs were obtained via bone marrow flush of the bilateral femurs. To extract cells, 10mL fat pads and 2mL of bone marrow was employed for the acquisition of ASCs and BMSCs, respectively. The solution containing ASCs was diluted five times prior to seeding and experimentation. All tissue was then cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic at 37°C under standard cell culture conditions. Once 80% confluence was achieved, the cells were trypsinized and passaged. Experimentation involved cells at passages three and four. Characterization of MSCs was confirmed via rat MSC Functional Identification kit (R&D Systems, Minneapolis, MN). Tri-lineage differentiation into adipocytes, osteocytes, and chondrocytes was confirmed via positive staining for FABP4, osteocalcin, and aggrecan, respectively (Figure 1).

Co-culture Model

Five cell lines from five rats were prepared. VFFs and MSCs were cultured separately for ~24 hours to ensure adequate cell health. VFFs (1.0×10^4) were then seeded on 6-well plates. ASCs and BMSCs (both at 1.0×10^4) were seeded on cell culture inserts with 0.4µm pores (Falcon, Corning, NY). Following 12 hours serum starvation (DMEM+0.1% Bovine Serum Albumin), the plates were rinsed with phosphate-buffered saline (PBS) and the cells were treated with serum-free DMEM+/-TGF-β1 (10ng/mL). The inserts containing MSCs were rinsed with PBS and gently transferred into 6-well plates containing VFFs. Control VFFs and MSCs were collected from the 6-well plates and from the inserts, respectively. Six experimental conditions were included: VFF control (VFF), VFFs with ASCs (VFF+ASC), and VFFs with BMSCs (VFF+BMSC) with or without TGF-β1. MSCs from the inserts were also harvested: ASC control (ASC), ASCs with VFFs (ASC+VFF), BMSC control (BMSC), and BMSCs with VFFs (BMSC+VFF) with or without TGF-β1. VFFs were individually co-cultured with ASCs or BMSCs from the same animal.

Cell Morphology

VFFs were imaged after 24 hours of co-culture using a Nikon Eclipse TS100 microscope (Nikon, Inc. Melville, NY) with an attached C-mount Morrell HDMI-02 camera (Morrell Instrument Company, Melville, NY; 10×).

Quantitative Real-Time Polymerase Chain Reaction

After six hours of co-culture, the inserts were removed and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quantity and quality of mRNA was evaluated via 260/280 ratio. RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction (PCR) employed the TaqMan Gene Expression kit (Life Technologies, Waltham, MA) and StepOne Plus (Applied Biosystems). qRT-PCR was performed to investigate changes in gene expression of ECM components, select components of the TGF- β signaling pathway, as well as HGF expression. To quantify the interaction between VFFs and MSCs, RNA from ASCs and BMSCs on the inserts was also extracted. The following genes were amplified: procollagen type I (*Col1a1*) and III (*Col3a1*), matrix metalloproteinase (*Mmp1a*), hyaluronan synthase 1 (*Has1*), *Has2* and *Has3*, *Smad3*, *Tgf- β 1*, TGF- β 1 receptor type I (*Tgf- β r1*) and type II (*Tgf- β r2*), and HGF (*Hgf*). The primer probes employed were: *Col1a1* (Rn01463848_m1), *Col3a1* (Rn01437681_m1), *Mmp1a* (Rn01486634_m1), *Has1* (Rn01455687_g1), *Has2* (Rn00565774_m1), *Has3* (Rn01643950_m1), *Smad3* (Rn00565331_m1), *Tgf- β 1* (Rn00572010_m1), *Tgf- β r1* (Rn00562811_m1), *Tgf- β r2* (Rn00579682_m1), *Hgf* (Rn00566673_m1), and *Gapdh* (Rn01775763_m1). All experiments included cells derived from five different animals run in triplicate. The Ct method was employed with GAPDH as the housekeeping gene.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

After 24 hours of co-culture, the inserts were removed and the plates were washed twice with cold PBS. VFFs were harvested via cell scraping and total cellular protein was extracted using the Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA), supplemented with Halt Protease inhibitor cocktail (Thermo Scientific, Waltham, MA), 0.5M EDTA Solution 100 $\times$ (Thermo Scientific, Waltham, MA), Calyculin A (Cell Signaling, Danvers, MA), and 2-mercaptoethanol (Life Technologies). Total protein was quantified via the Pierce 660nm Protein Assay (Thermo Scientific). Each protein lysate (0.5mg) was loaded on 8% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Protein was transferred to PVDF membranes (Invitrogen, Carlsbad, CA); the membranes were then blocked in nonfat milk overnight at 4°C. The membranes were incubated with a primary antibody against alpha smooth muscle actin (α -SMA) (Sigma, St Louis, MO; 1:10,000) or GAPDH (Cell Signaling, Danvers, MA; 1:1000) at 4°C overnight followed by one hour incubation with horseradish peroxidase-conjugated secondary antibody (Cell Signaling). Blots were visualized using chemiluminescence detection and quantified via ImageJ Software (National Institutes of Health; v1.41).

Statistical Analyses

For all analyses, the dependent variable of interest was subjected to a one way analysis of variance. If the main effect was significant at $p < 0.05$, post hoc comparisons were performed via the Tukey-Kramer method. Statistical significance was defined as $p < 0.01$ or $p < 0.05$ using StatView 5.0 (SAS Institute, Berkeley, CA). All data are expressed as means $\pm$ standard error.

RESULTS

Neither TGF- β 1 nor co-culture with MSCs altered VFF morphology

Cell morphology did not qualitatively change in response to TGF- β 1 or either co-culture condition (Figure 2).

Co-culture with MSCs suppressed pro-collagen and HAS mRNA expression in VFFs

To determine the effects of MSCs on VFFs, mRNA expression of ECM components (*Col1a1*, *Col3a1*, *Mmp1a*, *Has1*, *Has2*, and *Has3*) was quantified via RT-PCR. *Col1a1* and *Col3a1* decreased significantly in the VFF+BMSC groups with TGF- β 1 stimulation compared to control ($p<0.05$). *Col1a1* and *Col3a1* also decreased in VFF+ASC and VFF+BMSC without TGF- β 1 treatment. This decrease, however, did not achieve statistical significance (Figure 3A and 3B). *Mmp1a* trended towards increased expression VFFs in both co-culture environments, but did not achieve significance (Figure 3C). All *Has* subtypes were significantly reduced in VFF+BMSC response to TGF- β 1 (*Has1*- $p<0.01$, *Has2*- $p<0.05$, and *Has3*- $p<0.05$). *Has3* decreased significantly in VFF+ASC with TGF- β 1 treatment ($p<0.01$; Figure 3D-F).

VFFs had differential effects on ASCs and BMSCs in co-culture

Phenotypic changes to MSCs were quantified to determine the effects of VFFs with an emphasis on genes related to ECM metabolism. *Col1a1* and *Col3a1* gene expression decreased in ASC+VFF without TGF- β 1 stimulation (Figure 4A and B). *Mmp1a*, *Has1* and *Has2* mRNA significantly increased in BMSC+VFF regardless of TGF- β 1 stimulation. No effects were observed with regard to *Has3* expression in either co-culture condition or cell type (Figure 4C-F).

BMSCs suppressed TGF- β signaling and upregulated HGF in VFFs

To investigate whether MSCs affect TGF- β signaling, Smad3 and TGF- β 1 expression was quantified. In the absence of TGF- β 1, *Smad3* mRNA significantly decreased in VFF+BMSC compared to VFF control ($p<0.05$). With TGF- β 1 stimulation, however, *Smad3* and *Tgf- β 1* mRNA were significantly downregulated ($p<0.01$ and $p<0.05$, respectively), and this response was more pronounced in the VFF+BMSC compared to VFF+ASC (Figure 5A and B). To further characterize this response, mRNA for type I and II TGF- β receptors (*Tgf- β 1* and *Tgf- β 2*) was quantified. *Tgf- β 1* significantly decreased in VFF+BMSC when compared to VFF control or VFF+BMSC (both $p<0.05$) with TGF- β 1 stimulation. No significant differences were observed in *Tgf- β 1* expression in the absence of TGF- β 1 treatment. In addition, no changes in *Tgf- β 2* expression were observed across conditions or groups (Figure 5C and D). We further focused on fibrosis and quantified *Hgf* expression as it has been previously shown to be anti-fibrotic in the vocal folds.^{20,21} *Hgf* expression significantly increased in VFFs in both co-culture conditions regardless of TGF- β 1 treatment. This effect achieved statistical significance in the VFF+ASC and VFF+BMSC conditions without TGF- β 1 stimulation ($p<0.05$ and $p<0.01$, respectively) and in the VFF+BMSC condition with TGF- β 1 stimulation ($p<0.01$; Figure 5E).

MSCs decreased alpha-smooth muscle actin in VFFs

To confirm the characteristic change of VFFs to myofibroblasts, α -SMA protein levels were assessed. α -SMA increased in VFFs in response to TGF- β 1. This response, however, was blunted in both VFF+ASC and VFF+BMSC (Figure 6A). Quantification of these protein levels confirmed statistically-significant decreased α -SMA levels in VFF+ASC and VFF+BMSC with TGF- β 1 stimulation (both $p<0.01$), as well as in the absence of TGF- β 1 (both $p<0.05$; Figure 6B).

DISCUSSION

Vocal fold fibrosis continues to perplex clinicians. It is encouraging, however, that various therapeutic tools have shown efficacy in the laboratory. Among these novel tools, MSCs have gained increased attention across various fields given their capacity for tissue regeneration. MSCs are thought to contribute to the wound healing response via their inherent capacity to secrete growth factors, cytokines, and chemokines.²² We hypothesize that MSCs interact favorably with native vocal fold cells in the wound milieu. We employed a co-culture model to quantify the interactions between MSCs and VFFs *in vitro*. In addition, given the fundamental role of secreted TGF- β in injured tissue, our co-culture model included TGF- β 1 exposure to recapitulate the injured VF microenvironment. Clinically, MSC transplantation is typically autologous. As such, our co-culture model involved VFFs exposed to MSCs from the same animal. In this context, rats are an ideal animal model for this type of experimentation.

The tissue response following injury is typically described in three phases; inflammation, proliferation, and remodeling. ECM synthesis largely occurs during the proliferative phase. Collagen type III is produced as a temporary scaffold to encourage cell infiltration and is eventually replaced by collagen type I via proteolytic enzyme (MMP)-mediated biodegradation. Interestingly, vocal fold fibrosis differs from dermal scar with regard to increased collagen III expression well into the chronic reparative phase. This difference, in addition to increased collagen type I content, likely contributes to the altered biomechanical properties of scarred vocal folds.³ *Col1a1* and *Col3a1* mRNA expression significantly decreased in VFFs co-cultured with BMSCs under TGF- β 1 stimulation. Down-regulation of *Col1a1* and *Col3a1* mRNA expression did not achieve significance in this model without TGF- β 1 stimulation. Interestingly, both *Col1a1* and *Col3a1* expression was significantly down-regulated in co-cultured ASCs without TGF- β stimulation. These data likely suggest that collagen mRNA expression in VFFs was suppressed via paracrine BMSC-mediated signaling, especially in the context of TGF- β -stimulation. It is possible that VFF-mediated signaling suppressed collagen expression in ASCs in the absence of TGF- β . *Mmp1a* mRNA significantly increased in BMSCs co-cultured with VFFs regardless of TGF- β stimulation. These data likely indicate the initiation of enzymatic collagen degradation in our model.

HA is thought to be a key molecule in maintaining VF viscoelasticity.²³ Specifically, reduced HA in the human VF is correlated with changes to the viscoelastic properties.²⁴ *Has* is a HA synthesizing enzyme and each subtype has differing molecular weight and enzymatic activity.²⁵ *Has1* and *Has2* have been shown to be upregulated in acute rat VF healing, whereas *Has3* is upregulated in the more chronic phases of healing.²⁶ In the current

study, *Has2* mRNA expression significantly increased in VFF co-cultured with BMSCs with TGF- β 1 stimulation. In contrast, *Has1* and *Has3* expression in VFFs decreased in co-culture with both MSC types. With regard to bidirectional paracrine signaling, *Has1* and *Has2* expression significantly increased in BMSCs co-cultured with VFFs. These data likely indicate that HA synthesis is not only derived from existing cells in the wound bed including fibroblasts and myofibroblasts stimulated via paracrine MSC signaling, but from MSCs via autocrine or VFF-induced paracrine effects. Given that several previous *in vivo* studies demonstrated HA restoration with MSC transplantation,¹¹ HA secretion from transplanted MSCs via autocrine signaling could potentially compensate for paracrine signaling-mediated VFF inactivity. As noted previously, the primary histological change in vocal fold fibrosis is excessive accumulation of collagen and decreased HA. Our data suggest that preferable phenotypic changes are induced via interaction between VFFs and MSCs.

In scar, excessive ECM deposition and fibroblast-myofibroblast differentiation occur in parallel. Prolonged myofibroblast presence in the wound bed is associated with excessive collagen deposition, increased tissue stiffness, and irreversible fibrosis. TGF- β is a central mediator of myofibroblast differentiation and ECM deposition and has been implicated as a prime mediator of fibrosis across tissue systems. TGF- β signaling is complex and mediated largely via the Smad family of signaling proteins. Activated TGF- β receptor leads to phosphorylation of R-Smads (Smad2/Smad3). Phosphorylated Smad2/Smad3 then heterodimerize with Smad4 (Co-Smad) and translocate into the nucleus to regulate transcription.²⁷ Among these factors, Smad3 is thought to be a master regulator of fibrosis.²⁸ In the current study, *Smad3* mRNA expression in VFFs significantly decreased when co-cultured with BMSCs, with or without TGF- β stimulation. In addition, VFF expression of *Tgf- β 1* and *Tgf- β 1* decreased with *Tgf- β 1* stimulation when co-cultured with BMSCs. These data are consistent with previous work which described MSC-mediated suppression of R-Smads and endogenous TGF- β 1 in glomerular fibrosis *in vivo*.²⁹ VFF expression of α -SMA, a myofibroblast marker, decreased in co-culture with both ASCs and BMSCs. However, decreased TGF- β signaling genes were observed in VFFs co-cultured with BMSCs under of TGF- β stimulation, but decreased α -SMA expression may, in fact, be preferable as it would indicate inhibition of myofibroblast differentiation upstream of TGF- β signaling. In this study, we further investigated changes in HGF expression in our co-culture model. HGF expression was enhanced regardless of TGF- β 1 stimulation. Exogenous HGF was previously reported to suppress α -SMA expression in VFFs. This inhibitory effect on differentiation was putatively suggested to be related to the anti-fibrotic effects of HGF.³⁰ Data from the current study concur with these previous data; decreased α -SMA expression in VFFs under co-culture conditions may be related to HGF secretion as well as suppression of TGF- β signaling.

With regard to the source of MSCs, differences between the effects of ASCs and BMSCs on VFFs were observed. The magnitude of these effects tended to be greater with BMSCs; the paracrine effects of BMSCs were profound with regard to ECM regulation and suppression of TGF- β signaling. With regard to vocal fold fibrosis, ASCs were previously reported to hold increased therapeutic efficacy over BMSCs when combined with an atelocollagen scaffold.³¹ In other fields, contrary data exist.^{32,33} These differences could be related to the selection of experimental conditions including species, animal age, *in vitro* or *in vivo*

setting, and timing of harvest. Cumulatively, however, the emerging data regarding the utility of MSCs in the context of vocal fold fibrosis are encouraging.

CONCLUSION

MSCs favorably affected VFFs in the context of TGF- β 1 stimulation. Specifically, MSCs altered ECM metabolism, suppressed TGF- β signaling, and inhibited VFF myofibroblast differentiation. Although both ASCs and BMSCs had a favorable effect, BMSCs yielded more favorable effects on VFFs.

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Figure 1.

Representative immunofluorescent images of adipose-derived stem cells (ASCs; A-C) and bone marrow-derived stem cells (BMSCs; D-E) and differentiation into adipocyte, osteocyte, chondrocyte positive staining for FABP4 (A, D), osteocalcin (B, E), and aggrecan (C, F), respectively (Blue, DAPI; scale bar=50 μ m).

Figure 2.

Representative cell morphology of vocal fold fibroblasts (VFFs) 1 day after co-culture.

VFFs control (VFF: A,D), VFF co-cultured with adipose-derived stem cells (VFF+ASC: B, E) and VFF co-cultured with bone marrow-derived stem cells (VFF+BMSC: C, F) +/- TGF-β1 (10ng/mL; scale bar=50μm).

Figure 3.

VFF gene expression (mRNA) of extracellular matrix components in co-culture with adipose-derived stem cells or bone marrow-derived stem +/- TGF-β1 (10ng/mL). All genes were standardized to GAPDH expression and expressed as fold expression relative to control. Data presented as mean ± SEM of assays run in triplicate (n=5; ** $p < 0.01$, * $p < 0.05$). (A) *Col1a1* levels; (B) *Col3a1* levels; (C) *Mmp1a* levels; (D) *Has1* levels; (E) *Has2* levels; (F) *Has3* levels.

Figure 4.

Gene expression (mRNA) of extracellular matrix components in adipose-derived stromal cells and bone marrow-derived stromal cells co-cultured with VFFs +/- TGF-β1 (n=5; ** $p < 0.01$ compared with ASC control and †† $p < 0.01$ compared with BMSC control). (A) *Col1a1* levels; (B) *Col3a1* levels; (C) *Mmp1a* levels; (D) *Has1* levels; (E) *Has2* levels; (F) *Has3* levels.

Figure 5.

Gene expression (mRNA) for components of the TGF- β signaling pathway and hepatocyte growth factor in VFFs co-cultured with adipose-derived stromal cells or bone marrow-derived stromal cells +/- TGF- β 1 (n=5; ** p <0.01, * p <0.05). (A) *Smad3* levels; (B) *TGF- β 1* levels; (C) *TGF- β 1* levels; (D) *TGF- β 2* levels; (E) *Hgf* levels.

Figure 6.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis for α -SMA in VFFs co-cultured with adipose-derived stromal cells or bone marrow-derived stromal cells $\pm$ TGF- β 1; representative gel (A). GAPDH as the endogenous control and α -SMA protein expression was quantified relative to GAPDH (B). Data presented as mean $\pm$ SEM of assays run in triplicate (n=5; ** p <0.01, * p <0.05).