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Transplantation of immature hematopoietic progenitors expressing a leukemia inhibitory factor transgene influences muscle fibrosis in dystrophic mdx mice

Author

Vera Cruz, Maxine Faith Ramos

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Transplantation of immature hematopoietic progenitors expressing a leukemia inhibitory factor transgene influences muscle fibrosis in dystrophic mdx mice

A thesis submitted in partial satisfaction of the requirements

for the Master of Science in Physiological Science

by

Maxine Faith Ramos Vera Cruz

2020

ABSTRACT OF THE THESIS

Transplantation of immature hematopoietic progenitors expressing a leukemia inhibitory factor transgene influences muscle fibrosis in dystrophic mdx mice

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Maxine Faith Ramos Vera Cruz

Master of Science in Physiological Science

University of California, Los Angeles, 2020

Professor James G. Tidball, Chair

Current therapies for Duchenne muscular dystrophy (DMD) face major obstacles in achieving treatment efficacy. In this study, we tested a macrophage-mediated system to deliver a therapeutic molecule, called leukemia inhibitory factor (LIF), to dystrophic muscle in the *mdx* mouse model of DMD. Immature hematopoietic progenitors were transduced with a lentiviral vector containing a LIF transgene under control of the CD11b promoter. These modified immature hematopoietic progenitors were then transplanted into *mdx* mice so that donor-derived macrophages express LIF upon activation via the CD11b promoter sequence. We found differential effects on collagen accumulation in the diaphragm and quadriceps muscles. We also stimulated fibroblasts isolated from quadriceps and diaphragm muscles with LIF and found that LIF stimulation reduced collagen type III expression in fibroblasts from *mdx* quadriceps only.

These findings indicate that our strategy for delivery of potentially therapeutic transgenes to dystrophic muscle can influence muscle pathology but may vary between muscles.

The thesis of Maxine Faith Ramos Vera Cruz is approved.

Kenneth Dorshkind

V. Reggie Edgerton

James G. Tidball, Committee Chair

University of California, Los Angeles

2020

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Introduction

Duchenne muscular dystrophy (DMD) is a fatal disease caused by mutation of the gene that encodes the protein dystrophin. Dystrophin is a member of a protein complex that links the extracellular matrix (ECM) to the actin cytoskeleton in skeletal muscle fibers and cardiomyocytes.[1] When dystrophin is dysfunctional or absent, the structural integrity of the sarcolemma is compromised, and muscle fibers become susceptible to contraction-induced injury and suffer sarcolemma breakage.[2] Over the course of dystrophic pathology, muscles undergo repeated cycles of injury and regeneration, leading to increased fibrosis, pseudohypertrophy, chronic inflammation, and necrosis.[3] Cardiac and respiratory muscles like the diaphragm become severely affected, and inspiratory and cardiovascular capacities are dramatically impaired.[4] Consequently, most DMD patients die prematurely due to cardiac or respiratory failure.

When skeletal muscle is injured, immune cells infiltrate the injury site to begin tissue repair. Resident macrophages have been shown to release chemokines selectively that play a vital role in directing the migration of infiltrating neutrophils and monocytes to the injured muscle.[5] Neutrophils invade the muscle and release cytokines to recruit monocyte-derived macrophages, which then serve to phagocytose muscle debris and clear the injury site.[6] Macrophages take on two phenotypes during earlier versus later stages of the inflammatory response. The earlier stage is dominated by M1 macrophages that are classically activated by pro-inflammatory, T-helper (Th) 1 cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ).[7,8] M1 macrophages can release pro-inflammatory cytokines that can intensify the inflammatory response.[9] During later stages of inflammation, macrophages are alternatively activated into a more phenotypically-heterogeneous population of M2 macrophages. M2 macrophages are activated by anti-inflammatory, Th2 cytokines such as interleukin (IL) -4 and

IL-10.[9,10] These macrophages release anti-inflammatory cytokines and promote muscle regeneration.

In healthy muscle, inflammation eventually subsides, allowing the muscle to remodel so that myofibers mature and function is regained. However, in dystrophic pathology, repeated cycles of injury lead to chronic inflammation of the muscle and aberrant tissue remodeling, in which muscle is replaced with fibrous and fatty tissue.[11] M2 macrophages remain in the muscle in large numbers, and fibrosis, the scarring of tissue due to excessive accumulation of ECM components, occurs.[12,13]

In addition to mediating the inflammatory response, macrophages can play direct or indirect regulatory roles that affect the muscle's regenerative capacity.[14] For example, pro-inflammatory M1 macrophages express inducible nitric oxide synthase (iNOS) to generate cytotoxic levels of nitric oxide (NO). They can lyse muscle cells via an iNOS/NO-dependent mechanism, promoting further degeneration of myofibers.[9] M1 macrophages have also been shown to promote the proliferative capacity of satellite cells and myoblasts *in vitro*.[15,16]

M2 macrophages are made up of a more heterogeneous subset of phenotypes, characterized as M2a, M2b, and M2c, according to some nomenclatures.[17,18] M2a macrophages are activated by IL-4 and IL-13 and are associated with processes such as tissue repair, wound healing, and fibrosis.[9,10,19] Macrophages that shift from an M1 to an M2a phenotype show a reduction in iNOS expression and an increase in arginase 1 (Arg1) expression, demonstrating a shift in arginine metabolism in the muscle.[9] Whereas iNOS catalyzes the metabolism of arginine to generate citrulline and nitric oxide, Arg1 catalyzes the conversion of arginine to urea and L-ornithine, the precursor for proline which is used in collagen synthesis. Reductions in arginase expression have been shown to decrease fibrosis in dystrophic skeletal muscle, indicating that M2a

macrophages are closely linked to profibrotic processes during tissue remodeling.[20] M2b macrophages are activated by immune complexes and Toll-like receptors and release anti-inflammatory, Th2 cytokines such as IL-10.[21,22] M2c macrophages are activated by IL-10 and also release anti-inflammatory cytokines that deactivate the M1 phenotype and diminish inflammation in the muscle.[21] M2c, as well as M2a, macrophages can release profibrotic factors like transforming growth factor beta (TGFβ) to regulate the activity of fibroblasts in producing and remodeling ECM components.[23] Altogether, M2 macrophages are associated with anti-inflammatory and profibrotic processes in muscle repair.

Given their broad range of functions in the repair of injured muscle, macrophages are the most predominant population of inflammatory cells in dystrophic muscle with concentrations that can exceed 20,000 cells/mm^3 of muscle tissue during the acute peak of pathology.[24] Thus, due to their abundance in muscle during dystrophic pathology, macrophages can act as natural vectors to deliver a therapeutic agent to dystrophic muscle as a therapeutic approach. Various treatments are used today to slow the progression of dystrophic pathology. For example, corticosteroids are used to reduce inflammation in the muscle, leading to short-term improvements in muscle strength and function.[25] Other common palliative treatments include myostatin inhibitors to promote muscle regeneration, anti-fibrotic drugs to reduce excessive scarring, and phosphodiesterase (PDE) inhibitors to reduce muscle ischemia.[26,27] However, these treatments are administered systemically, posing issues in the specificity of the location and time of delivery of such agents. First, systemic delivery allows therapeutic agents to be delivered to non-muscle tissues, resulting in off-target effects. Second, the asynchronous progression of pathology in dystrophic muscles requires the timely modification of the delivery of therapeutic agents according to the severity of the pathology. By using macrophages as a natural vector, the therapeutic agent can be delivered to injury sites in dystrophic muscle and in high magnitudes when pathology is at its peak. When inflammation subsides, the number of

macrophages in the injury site declines as well, reducing the amount of therapeutic agent delivered to the muscle.

Macrophage overexpression of therapeutic agents has been shown to effectively diminish pathology in dystrophic muscle. Wehling-Henricks and Welc et al. demonstrated that macrophage overexpression of Klotho, a protein associated with anti-aging effects that is down-regulated in dystrophic muscle,[28,29] promotes myogenesis in *mdx* mice, the murine model of DMD.[30] Welc and Flores et al. also tested a similar macrophage-mediated delivery system with leukemia inhibitory factor (LIF), a pleiotropic, secreted cytokine belonging to the IL-6 family that has been shown to attenuate dystrophic pathology in *mdx* mice.[31] They found that macrophage overexpression of LIF reduced fibrosis in LIF transgenic *mdx* (LIF/*mdx*) mice and in *mdx* mice transplanted with LIF/*mdx* immature hematopoietic progenitors (LIF/*mdx* BMT).[32] These investigations demonstrate that macrophages can act as vectors to deliver a therapeutic molecule to dystrophic muscle in a targeted manner.

In this study, we sought to make this macrophage-mediated delivery system more translatable by transducing wild-type immature hematopoietic progenitors *ex vivo* to express a therapeutic transgene then transplanting them into *mdx* mice. We selected the *mdx* mouse as our model because it resembles the progression of DMD pathology, which includes acute onset followed by chronic inflammation and fibrosis.[33,34] To test our hypothesis, we selected LIF as our therapeutic agent because it has been shown to promote myogenesis and reduce fibrosis in *mdx* mice and promote myoblast proliferation by inhibiting apoptosis *in vitro*.[32, 35-40] We developed a lentiviral construct in which expression of a LIF transgene was placed under the control of the human CD11b promoter, an integrin highly expressed in differentiated and activated macrophages.[41,42] Previous investigations have shown that the CD11b promoter is capable of driving high-level expression of transgenes in murine macrophages.[43] Our approach

combines *ex vivo* transduction of immature hematopoietic progenitors with a transgene and transplantation of those modified cells into *mdx* mice to deliver a therapeutic molecule to sites with active inflammation via macrophages. These findings indicate that our strategy for delivery of potentially therapeutic molecules to dystrophic muscle can influence pathology.

Materials and Methods

Animals

All animal experimentation complied with guidelines established by the UCLA Animal Research Committee. *Mdx* and C57BL/6J mice were purchased from the Jackson Laboratory and bred and housed in a pathogen-free vivarium.

Mdx mice that were transplanted with transduced immature hematopoietic progenitors were sacrificed at three and six months post-transplantation. The hind limb muscles of one leg and one half of the diaphragm were frozen in liquid nitrogen-cooled isopentane for histology. The hind limb muscles of the contralateral leg and the contralateral hemidiaphragm were frozen in liquid nitrogen for either qPCR or Western blot assay. Aliquots of bone marrow cells (BMCs) were used to determine the proportion of total BMCs that were donor-derived (the level of chimerism) assayed by fluorescent in situ hybridization of the Y-chromosome (Kreatech FISH Probes).

Transduction and transplantation of immature hematopoietic progenitors

Self-inactivating integrating lentiviral vectors were made through the Integrated Molecular Technologies Core at UCLA. Expression of the LIF construct was validated in murine macrophages. The lentiviral vectors for the LV.GFP and LV.LIF mice contain a CD11b promoter sequence and a histone-fused fluorescent reporter. The lentiviral vector for the LV.LIF group also contains a T2A self-cleaving peptide sequence and the LIF transgene downstream of the CD11b promoter (Figure 1A).

Immature, lineage negative (lin-) hematopoietic progenitors were separated from total BMCs obtained from six- to ten-week-old, male, wild-type mice, using magnetic-activated cell sorting

(Miltenyi Biotec) with biotinylated antibodies against CD5, CD45R, CD11b, Gr-1, 7-4, and Ter-119. To enhance cell transduction, the lin- cells were cultured in tissue culture plates coated with recombinant human fibronectin (Retronectin, Clontech). The cells were grown in a pre-stimulation medium containing IL-3, stem cell factor, thrombopoietin, and fms-like tyrosine kinase-3 ligand to promote stem cell viability. The lentiviral vectors were added to the cell cultures four hours after pre-stimulation then incubated overnight.

We administered a trimethoprim sulfamethoxazole regimen to female, three- to four- month old recipient mice to prepare them for transplantation of the transduced immature hematopoietic progenitors and reduce the likelihood of infection occurrence. On the day prior to transplantation, the recipient mice were subjected to irradiation (two doses of 450 Rad, three hours apart). 1.5×10^5 of the transduced lin- progenitors were intravenously injected into the mice. This number of cells was sufficient to reconstitute the mouse's hematopoietic lineage. The LV.LIF group of recipient mice received immature hematopoietic progenitors transduced with the LIF transgene, while the LV.GFP group received immature hematopoietic progenitors without the LIF transgene.

Validation of lentiviral construct expression via green fluorescent protein immunohistochemistry

Cross sections of diaphragm and quadriceps tissues from transplantation recipient *mdx* mice were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for five minutes then washed with PBS. Sections were incubated in 0.3% hydrogen peroxide in PBS for ten minutes then washed with PBS. The cross sections were blocked with a blocking buffer solution (3% bovine serum albumin (BSA), 0.05% Tween-20, 0.18% gelatin, 0.09% NaN_3, 0.8% NaCl, and 0.05% Tris base) for one hour and subsequently washed with PBS. The sections were then incubated for three hours at room temperature with an anti-green fluorescent protein (GFP) antibody (Abcam, ab13970) diluted at 1:500 in an antibody buffer solution (3% BSA, 0.18%

gelatin, 0.09% NaN_3, 0.8% NaCl, and 0.05% Tris base). Following the primary antibody incubation, the sections were washed again in PBS and then incubated in biotinylated goat anti-chicken antibody diluted in PBS (Vector, 1:200) for thirty minutes. Secondary antibody incubation was followed by a PBS wash. Sections were then incubated in avidin horseradish peroxidase (Vector) diluted in PBS (1:1000) for thirty minutes. Sections were washed in PBS for a final time then developed with an A.E.C. Peroxidase Substrate Kit (Vector) for ten minutes. We then confirmed by immunohistochemistry that inflammatory lesions in the muscles of *mdx* mice receiving transduced cells were enriched in GFP+ mononucleated cells (Figure 1B).

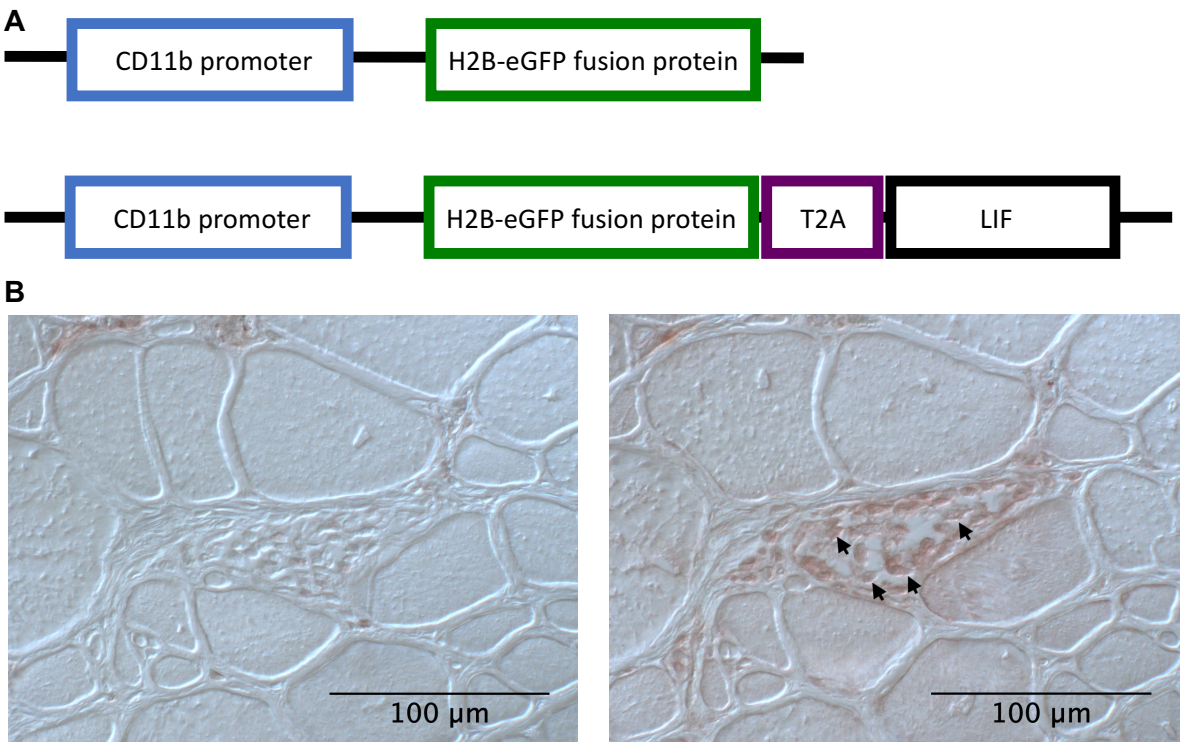


Figure 1. Schematic and validation of lentiviral constructs for transduction of immature hematopoietic progenitors. **A)** Top: Lentiviral construct of control (LV.GFP) *mdx* mice contain the human CD11b promoter, which increases in expression during differentiation and activation of myeloid cells, and the H2B-eGFP fusion protein, which encodes a nuclear localized fluorescent reporter. Bottom: Lentiviral construct of LIF-treated (LV.LIF) *mdx* mice contain the human CD11b promoter; H2B-eGFP fusion protein; T2A, a self-cleaving peptide sequence; and the LIF transgene. **B)** Cross-sections of quadriceps muscles from LV.GFP *mdx* mice at six months post-transplantation were immunolabeled with anti-GFP. Left: negative control without anti-GFP antibody, right: positively labeled cross-section. Black arrows indicate inflammatory cells in lesioned area.

Collagen deposition

We assayed for accumulation of collagen types I and III in diaphragm and quadriceps muscles that were sectioned at a thickness of 10 μm. The cross sections were fixed in cold acetone for ten minutes and then allowed to air dry for ten minutes. Sections were rehydrated with a PBS wash. The sections were then incubated in 0.3% hydrogen peroxide in PBS for ten minutes. Sections were again washed in PBS. Sections were blocked with a blocking buffer solution (3% BSA, 0.05% Tween-20, and 0.18% gelatin in PBS) for thirty minutes. The sectioned tissues were subsequently washed with PBS. The sections were then incubated overnight at 4ºC in either goat anti-collagen type I (Southern Biotech, 1:50) or goat anti-collagen type III (Southern Biotech, 1:50) diluted in the blocking buffer solution. Following the primary antibody incubation, the sections were washed in PBS. The sections were incubated in biotinylated horse anti-goat antibody diluted in PBS (Vector, 1:200) for thirty minutes, followed by a PBS wash. The sections were then incubated in streptavidin-horseradish peroxidase (HRP) diluted in PBS (1:1000) for thirty minutes, followed by a final PBS wash. The sections were then developed with an A.E.C. Peroxidase Substrate Kit (Vector) for eight minutes.

Collagen accumulation was quantified in sections observed through an Olympus BX50 microscope at 20X magnification and overlaid with a 10x10 grid. The number of total intercepts overlying tissue in the field and the number of intercepts overlying tissue positively stained with collagen types I or III were counted. The number of collagen-positive intercepts was divided by the total number of intercepts to calculate percent area of fibrosis.

Fibrotic cell quantification and imaging

We quantified the number of fibrotic cells in muscle sections using antibodies to the collagen chaperone, heat shock protein 47 (HSP47). Cross sections of diaphragm and quadriceps

tissues from transplantation recipient *mdx* mice were fixed in cold acetone for ten minutes and then allowed to air dry for ten minutes. Sections were washed with PBS then treated with TrueBlack® (diluted 1:20 in 70% ethanol) for five minutes. Sections were washed three times with PBS then blocked with a blocking buffer solution (3% BSA, 0.18% gelatin, 0.09% NaN_3, 0.8% NaCl, and 0.05% Tris base) for one hour at room temperature. The sections were subsequently washed with PBS then incubated overnight at 4ºC with a recombinant anti-HSP47 antibody (Abcam, ab109117) diluted 1:100 in the blocking buffer. Following the primary antibody incubation, the sections were washed in PBS. Sections were incubated with DyLight 594 goat anti-rabbit IgG antibody in PBS (Vector, 1:200) for one hour followed by three PBS washes. The sections were mounted with ProLong™ Gold Antifade Mountant with DAPI and cover slipped.

The immunostained sections were quantified at 40X magnification using a Leica DM RXA microscope. All HSP47+ cells were counted, excluding those located in the epimysium. For diaphragm tissue quantification, the entire section was observed. For quadriceps tissue quantification, 250 fields were counted.

We imaged the fibrotic cells by following the same immunostaining protocol with a slight modification: the primary antibody solution contained recombinant anti-HSP47 antibody (Abcam, ab109117) diluted at 1:100 and propidium iodide (Trevigen) diluted at 1:50 in the blocking buffer solution.

Fibroblast isolation

Fibroblasts were isolated from six-month-old *mdx* and wild-type mice. Quadriceps and diaphragm muscles were dissected and rinsed in PBS. Muscles were minced into a fine slurry then digested in 3 ml of enzyme buffer (Dulbecco's Modified Eagle Medium (DMEM, Sigma), 1% penicillin/streptomycin (P/S), 2.4 U/ml dispase II (Invitrogen), and 1% collagenase, type II

(Gibco)) per gram of muscle at 37°C for forty minutes with gentle shaking and frequent trituration. Sample volumes were raised to 10 ml with DMEM and passed through 100 μm cell strainers (Falcon). The digestates were then pelleted at 350 g for five minutes. Cells were resuspended in 5 ml of fibroblast isolation medium (DMEM, 1% P/S, and 10% fetal bovine serum (FBS)) and pre-plated in collagen- and gelatin-coated culture dishes. The cells were then incubated at 37°C in 5% CO_2 for one hour to allow fibroblasts to adhere to the plate. After the one-hour incubation, the plates were washed with DPBS to remove non-adherent cells, and 3 ml of fibroblast growth medium were added (DMEM, 1% P/S, and 20% FBS). The cells were incubated at 37°C in 5% CO_2 and fed three times a week with fresh fibroblast growth medium until confluent. When cells were 70-80% confluent, they were stimulated with either 10 ng/ml of LIF recombinant protein (eBioscience, 14-8521-80) or 0.1% BSA for twenty-four hours.

RNA isolation

RNA was isolated from 50-100 mg of frozen diaphragm or quadriceps muscles after homogenization in Trizol (Life Technologies). Each tissue sample underwent at least three, thirty-second bursts of homogenization with each period followed by thirty seconds of cooling on ice. Homogenized samples were centrifuged at 12,000 g for ten minutes at 4°C to pellet insoluble material. The supernatant containing the RNA was incubated at room temperature for five minutes to allow the dissociation of nucleoprotein complexes. Chloroform at 20% of the original Trizol volume was added to each sample and incubated for three minutes at room temperature. The samples were centrifuged at 12,000 g for fifteen minutes at 4°C to separate them into three distinct layers: organic, phenol-$CHCl_3$ phase (red and located at the bottom), protein phase (white and located in the middle), and aqueous RNA phase (clear and located at the top). The top aqueous layer containing the RNA was carefully drawn and transferred into a new tube, where an approximately equal volume of isopropyl alcohol was added. The samples

were mixed by inversion then incubated at room temperature for ten minutes. They were then centrifuged at 12,000 g for fifteen minutes at 4°C, resulting in an RNA pellet. The pellet was resuspended in 1 ml of 75% ethanol and centrifuged at 12,000 g for 10 minutes at 4°C. After centrifugation, the supernatant was removed, and the pellet air dried on ice. Once dry, the pellets were resuspended in RNase-free water. RNA samples were purified with RNeasy Mini Kit (Qiagen). RNA concentrations were determined using a Beckman DU730 spectrophotometer. All samples had a 260/280 ratio greater than 1.8, and RNA quality was assessed via 1.2% agarose gel.

To isolate RNA from fibroblasts, fibroblast growth medium was removed and replaced with 1 ml of Trizol per sample after 24-hour stimulation with BSA or LIF. Cells were detached with a cell scraper and lysed using a 23-gauge syringe. All subsequent steps for RNA isolation from the centrifugation to pellet insoluble material until resuspension of RNA pellets with RNase-free water were conducted as stated above. RNA from fibroblasts isolated from quadriceps muscles were purified with RNeasy Mini Kit (Qiagen), and RNA from fibroblasts isolated from diaphragm muscles were purified with RNA Clean & Concentrator-5 Kit (Zymo), according to manufacturer instructions. RNA concentrations were determined using a Beckman DU730 spectrophotometer. All samples had a 260/280 ratio greater than 1.9, and RNA quality was assessed via 1.2% agarose gel.

Quantitative real-time polymerase chain reaction (qPCR)

To generate cDNA, samples with 1 μl of Oligo(dT)$_{12-18}$ primer (Invitrogen), 1 μg of RNA, and autoclaved water were heated at 65°C for five minutes then chilled on ice. A master mix of 5X First-Strand Buffer (Invitrogen), 0.1 M DTT (Invitrogen), and 10 mM dNTPs was added to the samples then incubated at 42°C for two minutes. SuperScript™ II RT (Invitrogen) was added to

each sample. The samples were then incubated at 42°C for 50 minutes then at 70°C for fifty minutes to heat inactivate the enzyme. The final cDNA sample was diluted 1:1 in autoclaved water and stored at -20°C.

Two formats were used for qPCR analysis: 96-well and 384-well plates. For the 96-well format, each well contained 2 μl of cDNA and 23 μl of a master mix (autoclaved water, SYBR® Green Supermix (Bio-Rad), and the corresponding forward and reverse primers). Reference genes used were Rnps1 and Srp14 for diaphragm tissues and Rplo and Tpt1 for quadriceps tissues. For the 384-well plate, each well contained 2 μl of cDNA and 8 μl of a master mix (autoclaved water, SYBR green enzyme, and the corresponding forward and reverse primers). Reference genes used were Rnps1 and Tpt1 for fibroblasts.

Statistical analysis

All statistical analyses were performed using unpaired Student t-tests in Prism with differences considered significant at $p < 0.05$. All graphs display mean ± sem.

Results

Collagen expression in diaphragm and quadriceps muscles

Because the diaphragm is one of the most severely affected muscles in DMD pathology,[4,34] we assayed for fibrosis by measuring the percentage of the volume of diaphragm muscles positive for collagen. At three months post-transplantation, we observed no difference in percent volume of collagen types I and III between the LV.GFP and LV.LIF groups (Figures 2A,B). At six months post-transplantation, no difference was observed in collagen type I percent volume between the LV.GFP and LV.LIF mice (Figure 2C). However, percent volume of collagen type III was reduced in the LV.LIF *mdx* mice (Figures 2D,E). Due to the large decrease in collagen type III percent volume at six months post-transplantation, we assayed the rate of accumulation of collagen type III from the time of transplantation to six months after treatment. We observed a slower rate of accumulation in collagen type III from the time of transplantation to six months post-transplantation in the LV.LIF *mdx* mice (Figure 2F).

We also measured the relative mRNA expression of collagens type I and III via qPCR analysis. We found a downward trend in collagen type I expression ($p = 0.08$) and a significant reduction in collagen type III expression in the diaphragm muscles of our LV.LIF *mdx* mice at three months post-transplantation, but no difference was observed at six months post-transplantation (Figures 3A-D).

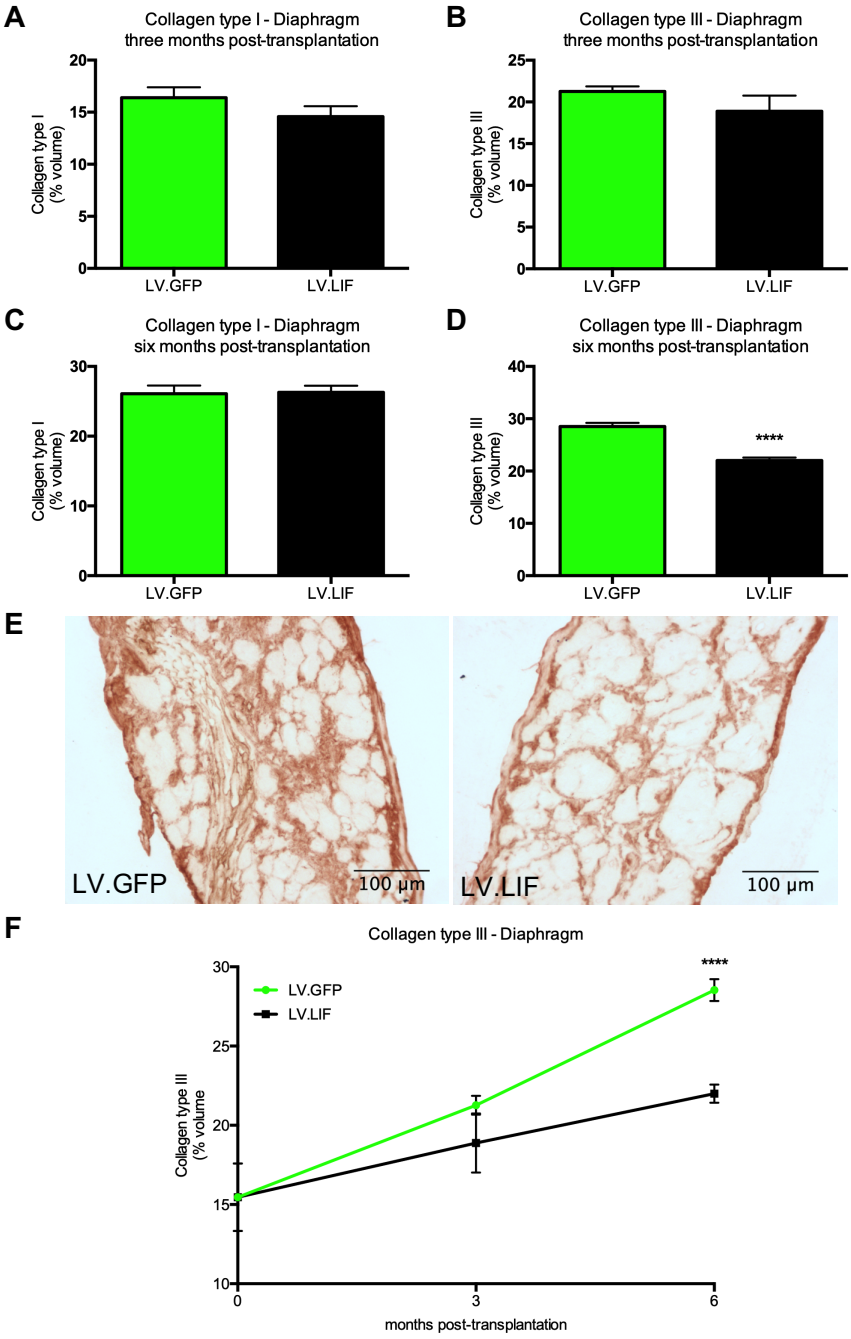


Figure 2. Macrophage-mediated overexpression of LIF slows the rate of collagen type III deposition in the diaphragm of *mdx* mice. **A-D)** Percent volume analysis of diaphragm muscles from LV.GFP and LV.LIF *mdx* mice three and six months post-transplantation. Cross-sections were immunolabeled with anti- collagen type I and collagen type III. (n = 5 for LV.GFP three months post-transplantation, n = 6 for LV.GFP six months post-transplantation, n = 6 for all LV.LIF except for collagen type III at six months-post transplantation (n=5); **** indicates $p < 0.0001$) **E)** Cross-sections of diaphragm at six months post-transplantation immunolabeled with anti-collagen type III. (LV.GFP, left; LV.LIF, right) **F)** Time course analysis of percent volume of collagen type III in diaphragm muscles from LV.GFP and LV.LIF *mdx* mice. (n = 6, 0 months post-transplantation)

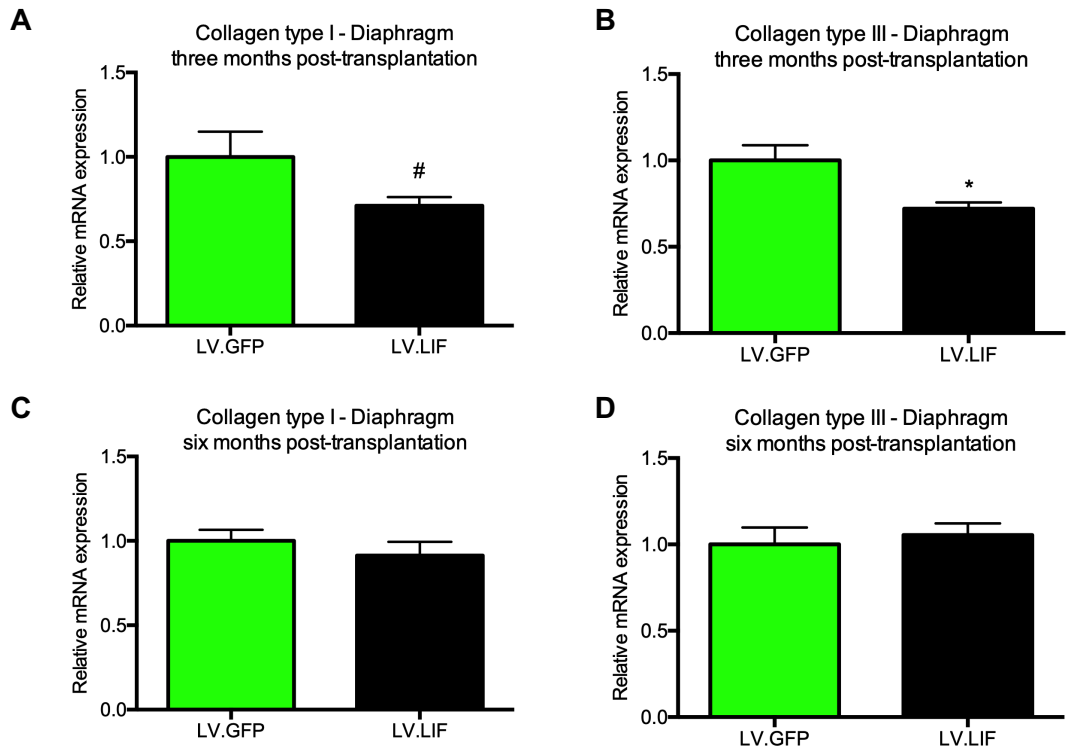


Figure 3. Relative mRNA expression of collagen types I and III are reduced in the diaphragm of LV.LIF *mdx* mice at three months post-transplantation. **A-D)** qPCR analysis of diaphragm muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of collagen types I and III at three (A,B) and six (C,D) months post-transplantation. (n = 6 for all groups except for LV.GFP groups at three months post-transplantation (n=5); # indicates $p < 0.1$, * indicates $p < 0.05$)

Given that the progression of pathology is vastly different in limb skeletal muscles of *mdx* mice compared to that in the diaphragm,[44-46] we also assayed for fibrosis in the quadriceps muscles of our LV.LIF *mdx* mice. At three months post-transplantation, there was a downward trend in the percent volume of collagen type I ($p = 0.07$), but no difference was observed in percent volume of collagen type III in the quadriceps muscles of the LV.LIF group (Figures 4A,B). However, at six months post-transplantation, we observed an upward trend in the percent volume of both collagen types I and III in the quadriceps muscles of the LV.LIF group ($p = 0.08$ for both) (Figures 4C-E). QPCR analysis showed no difference between the two groups at three and six months post-transplantation (Figures 5A-D).

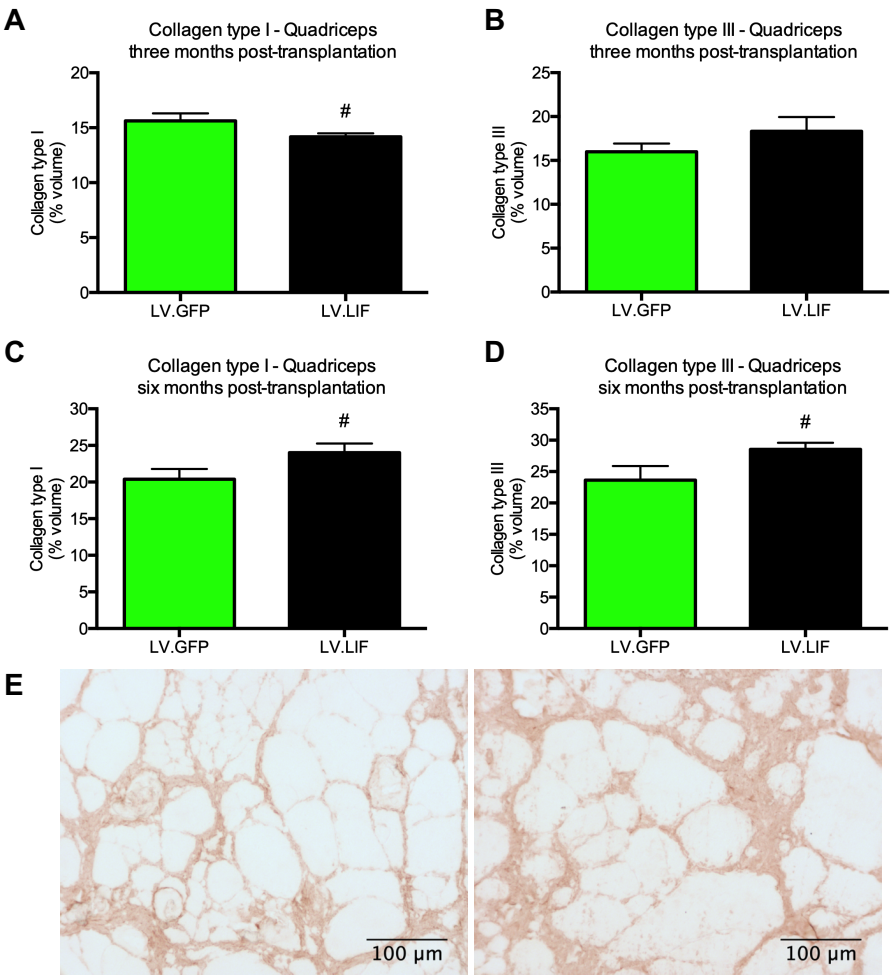


Figure 4. Collagen deposition in quadriceps does not differ significantly in LV.GFP and LV.LIF *mdx* mice. **A-D)** Percent volume analysis of quadriceps muscles from LV.GFP and LV.LIF *mdx* mice three and six months post-transplantation. Cross-sections were immunolabeled with anti-collagen type I and collagen type III. (n = 6 for all groups except for LV.GFP groups at three months post-transplantation (n=5); # indicates $p < 0.1$) **E)** Cross-sections of quadriceps muscles at six months post-transplantation immunolabeled with anti-collagen type III. (LV.GFP, left; LV.LIF, right)

Collagen production

We tested whether the differences that we observed in collagen accumulation in the LV.LIF *mdx* mice were attributable to differences in the numbers of collagen producing cells by assaying HSP47 expression as a marker for collagen producing cells. During collagen synthesis, HSP47 acts as a molecular chaperone by binding procollagens to prevent their unfolding and aggregation as they are transferred from the endoplasmic reticulum to the Golgi apparatus.[47] In

both diaphragm and quadriceps muscles, we observed no difference in the number of HSP47+ cells at three and six months post-transplantation, although there was a strong trend for fewer HSP47+ cells in LV.LIF quads at three months post-transplantation (Figures 6A-F). We also found no difference in expression levels of Serpinh1, the gene that encodes HSP47, at both three and six months post-transplantation in diaphragm muscles (Figures 6G-H). In the quadriceps muscles, we found no difference at three months post-transplantation, but at six months post-transplantation, we observed a significant increase in Serpinh1 expression levels in the LV.LIF group (Figures 6I-J).

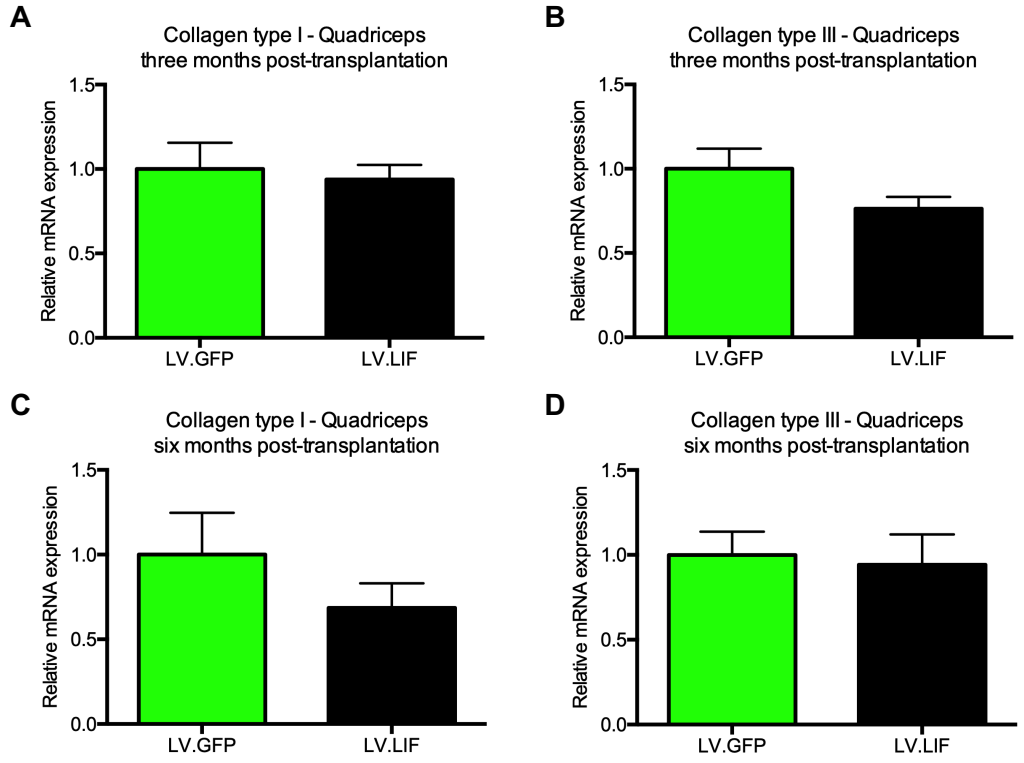
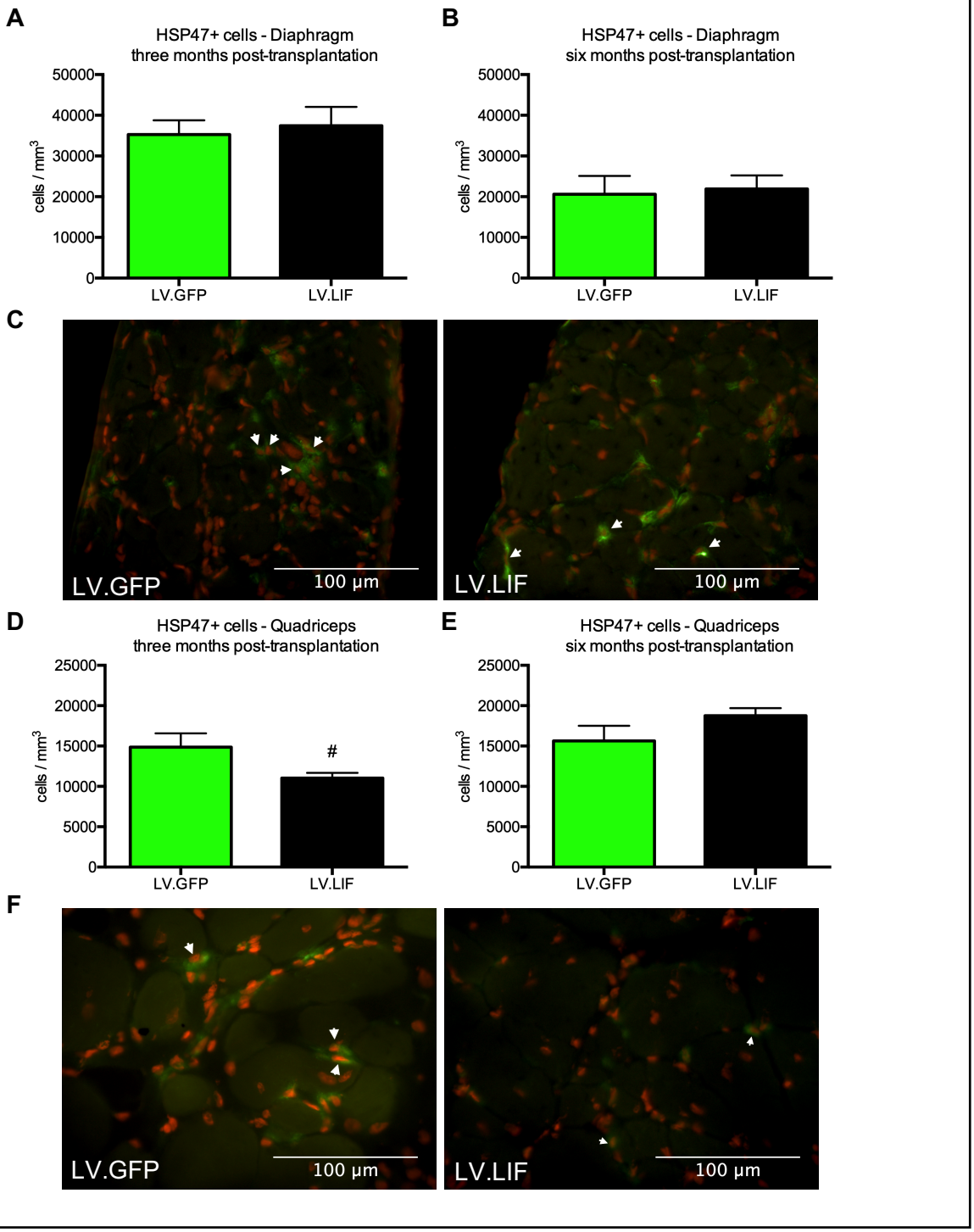


Figure 5. Relative mRNA expression of collagen types I and III do not change in the quadriceps of LV.LIF *mdx* mice. **A-D)** qPCR analysis of quadriceps muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of collagen types I and III at three (A,B) and six (C,D) months post-transplantation. (n = 5 for LV.GFP at three months post-transplantation, n = 6 for LV.LIF at three months post-transplantation, n = 7 for LV.GFP at six months post-transplantation, n = 5 for LV.LIF at six months post-transplantation)


A
HSP47+ cells - Diaphragm
three months post-transplantation
cells / mm3
50000
40000
30000
20000
10000
0
LV.GFP
LV.LIF
B
HSP47+ cells - Diaphragm
six months post-transplantation
cells / mm3
50000
40000
30000
20000
10000
0
LV.GFP
LV.LIF
C
LV.GFP
100 µm
LV.LIF
100 µm
D
HSP47+ cells - Quadriceps
three months post-transplantation
cells / mm3
25000
20000
15000
10000
5000
0
#
LV.GFP
LV.LIF
E
HSP47+ cells - Quadriceps
six months post-transplantation
cells / mm3
25000
20000
15000
10000
5000
0
LV.GFP
LV.LIF
F
LV.GFP
100 µm
LV.LIF
100 µm

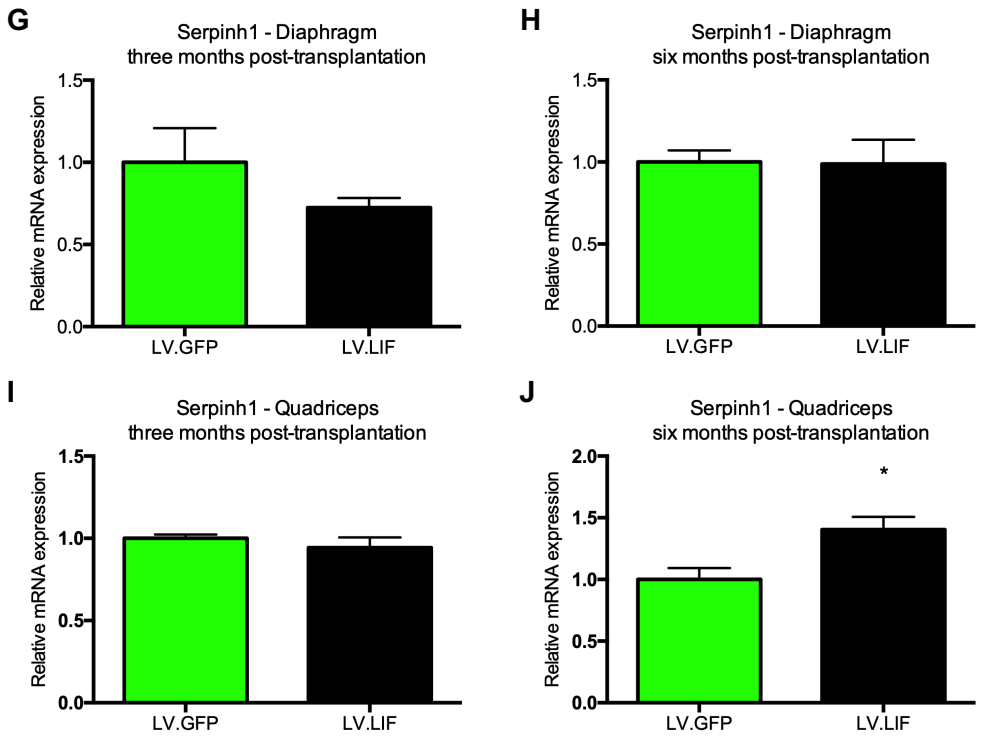


Figure 6. *Ex vivo* transduction and transplantation of immature hematopoietic progenitors to drive LIF overexpression in macrophages does not affect the number of HSP47+ cells but increases relative mRNA expression levels in the quadriceps. **A,B)** Number of HSP47+ cells in the diaphragm muscles of LV.GFP and LV.LIF *mdx* mice at three and six months post-transplantation normalized to muscle volume. (n = 6 for all except LV.GFP at three months post-transplantation) **C)** Representative images of diaphragm muscles from LV.GFP (left) and LV.LIF (right) *mdx* mice at three months post-transplantation with anti-HSP47 (green) and propidium iodide (red). Arrows indicate HSP47+ cells. **D,E)** Number of HSP47+ cells in the quadriceps muscles of LV.GFP and LV.LIF *mdx* mice at three and six months post-transplantation normalized to muscle volume. (n = 5 for both groups at three months post-transplantation, n = 7 for LV.GFP and n = 6 for LV.LIF at six months post-transplantation; # indicates $p < 0.1$) **F)** Representative images of quadriceps muscles from LV.GFP (left) and LV.LIF (right) *mdx* mice at three months post-transplantation with anti-HSP47 (green) and propidium iodide (red). **G-J)** qPCR analysis of diaphragm (G,H) and quadriceps (I,J) muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of Serpinh1 at three (G,I) and six (H,J) months post-transplantation. (n = 5 for LV.GFP, n = 6 for LV.LIF of diaphragm groups; n = 3 for both LV.GFP, n = 6 and n = 5 for LV.LIF at three and six months post-transplantation, respectively, in quadriceps groups; * indicates $p < 0.05$)

We also measured mRNA expression levels of Arg1 and Arg2 because these enzymes have been shown to affect fibrotic development in dystrophic muscle.[20] In the diaphragm, there were trends for increased Arg2 expression in LV.LIF *mdx* mice at three months post-transplantation ($p = 0.06$) and decreased Arg1 expression at six months post-transplantation ($p = 0.13$) (Figures

7A-D). In the quadriceps, there were weak trends for reduced Arg1 expression at both three and six months post-transplantation (p = 0.34; p = 0.27) (Figures 7E-H).

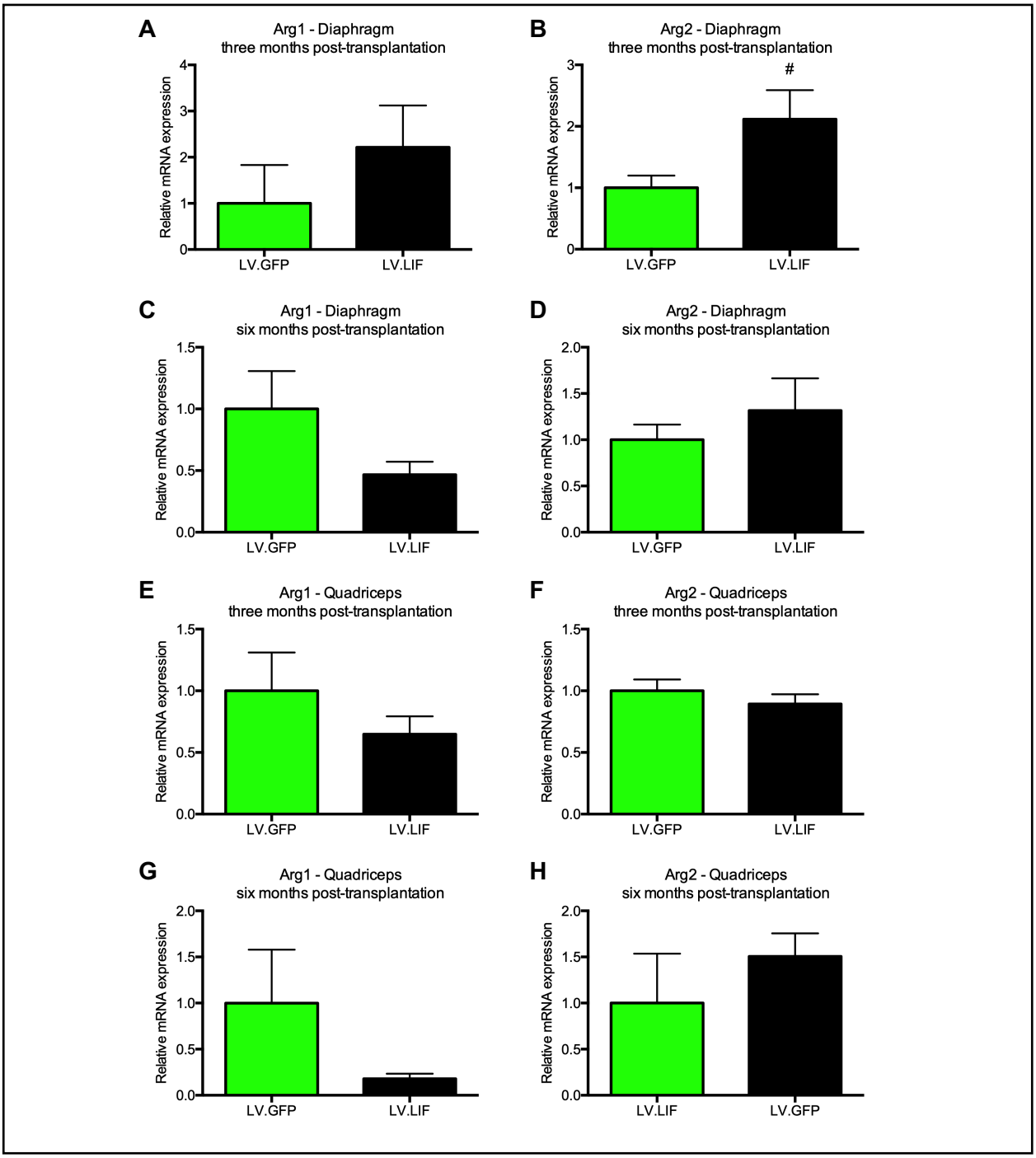


Figure 7. Arginase expression is unaffected in the quadriceps muscles but shows a trend for reduction in the diaphragms of LV.LIF *mdx* mice. **A-D)** qPCR analysis of diaphragm muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of Arg1 and Arg2 at three (A,B) and six (C,D) months post-transplantation. (n = 5 for all groups at three months post-transplantation except for LV.LIF group for Arg1 analysis (n = 6), n = 6 for all groups at six months post-transplantation except for LV.LIF group for Arg2 analysis (n = 4); # indicates $p < 0.1$) **E-H)** qPCR analysis of quadriceps muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of Arg1 and Arg2 at three (E,F) and six (G,H) months post-transplantation. (n = 5 for LV.GFP and n = 6 for LV.LIF at three months post-transplantation, n = 7 and n = 6 for LV.GFP groups for Arg1 and Arg2 analyses respectively, at six months post-transplantation, n = 5 for LV.LIF at six months post-transplantation)

Extracellular matrix breakdown

We also assayed for expression of enzymes that affect ECM breakdown to further examine the causes of the *in vivo* differences we observed in collagen accumulation. We measured the relative mRNA expression of two matrix metalloproteinases (MMP), MMP2 and MMP9, which degrade various collagens in the extracellular matrix.[48] MMP2 and MMP9, particularly, have been shown to be involved in inflammatory and profibrotic processes in dystrophic muscles of the canine model of DMD.[49] We also measured the relative mRNA expression levels of two tissue inhibitors of metalloproteinases (TIMP), TIMP1 and TIMP2, which inhibit the activity of MMPs.[50] In the diaphragm, MMP9 expression tended to be higher at three months post-transplantation ($p = 0.06$) (Figure 8A). At six months post-transplantation, TIMP1 expression levels tended to be lower ($p = 0.06$) (Figure 8B). In the quadriceps muscles, MMP2 expression levels tended to be reduced ($p = 0.07$) at three months post-transplantation, and no difference was observed in the expression levels of MMPs and TIMPs at six months post-transplantation (Figures 8C-D).

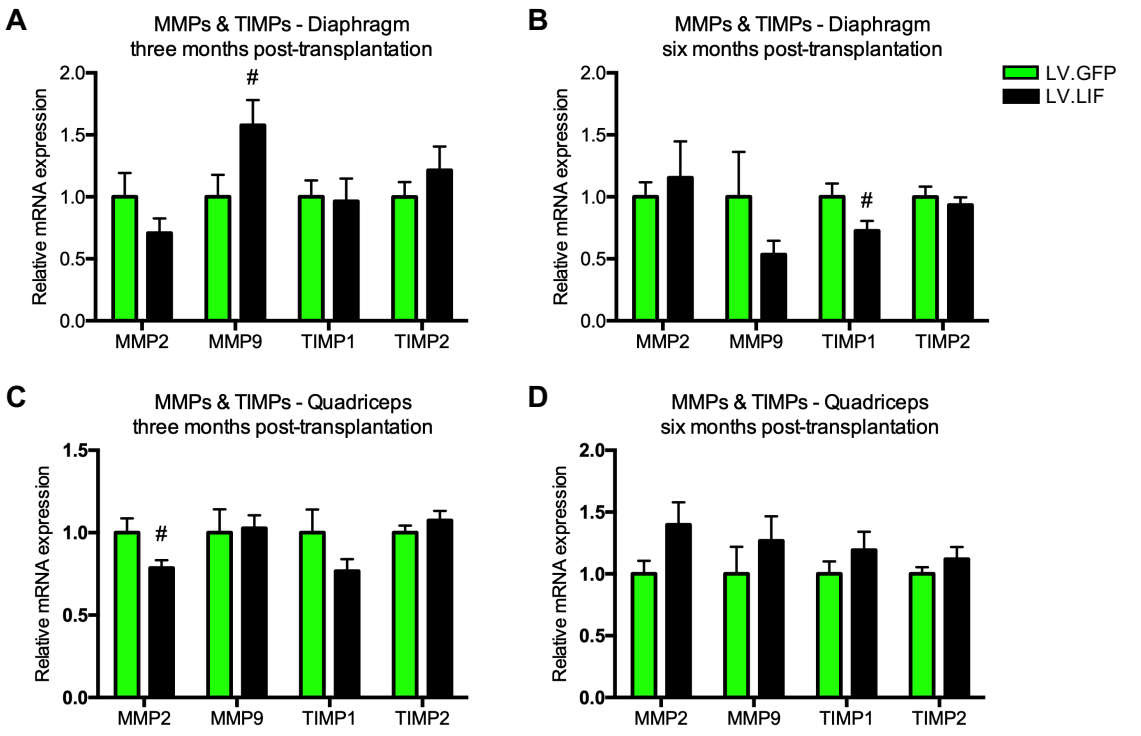


Figure 8. Macrophage-mediated delivery of LIF does not affect expression of ECM remodeling genes. **A,B)** qPCR analysis of diaphragm muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of MMP2, MMP9, TIMP1, and TIMP2 at three (A) and six (B) months post-transplantation. (n = 5 for all LV.GFP except for MMP2 and MMP9 analyses at six months post-transplantation; n = 6 for all LV.LIF; # indicates $p < 0.1$) **C,D)** qPCR analysis of quadriceps muscles from LV.GFP versus LV.LIF *mdx* mice show relative mRNA expression levels of MMP2, MMP9, TIMP1, and TIMP2 at three (C) and six (D) months post-transplantation. (n = 3 for all LV.GFP except for MMP2 at three months post-transplantation (n = 2), n = 5 for all LV.LIF except for MMP2 and TIMP2 at three months post-transplantation (n = 6); # indicates $p < 0.1$)

Fibroblast response to LIF *in vitro*

LIF has been shown to reduce collagen production and MMP activity in fibroblast cultures.[51] Therefore, we compared the potential effects of LIF on collagen expression in fibroblasts derived from diaphragm and quadriceps muscles. We measured the relative mRNA expression of collagen in fibroblasts isolated from quadriceps and diaphragm muscles of six-month-old wild-type and *mdx* mice after 24-hour stimulation with 10 ng/μl of LIF. We found that unstimulated fibroblasts isolated from *mdx* quadriceps muscles expressed higher levels of collagen type III than those from wild-type quadriceps muscles and LIF stimulation decreased their collagen type III expression back to wild-type levels (Figure 9A). We also observed a downward trend in

expression of collagen type I in fibroblasts isolated from wild-type quadriceps muscles after LIF stimulation (Figure 9B). LIF had no effect on collagen type III expression by fibroblasts isolated from diaphragm muscles of either wild-type or *mdx* mice (Figure 9C). However, we observed an upward trend in expression of collagen type I after LIF stimulation in fibroblasts isolated from wild-type diaphragm muscles (Figure 9D).

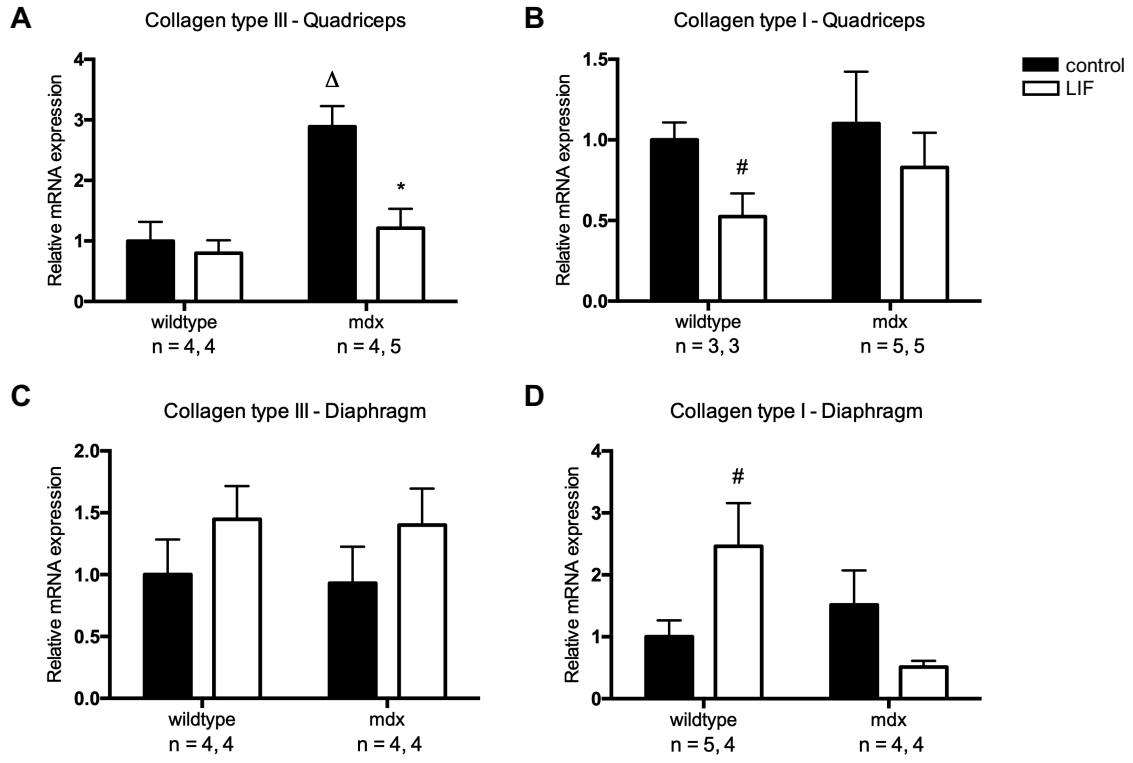


Figure 9. LIF stimulation decreases collagen type III expression in fibroblasts isolated from quadriceps muscles of *mdx* mice. **A-D)** qPCR analysis of collagen types I and III expression by fibroblasts isolated from quadriceps (A,B) and diaphragm (C,D) muscles of six-month-old wild-type and *mdx* mice. Fibroblasts were stimulated with 0.1% BSA (control) or 10 ng/ml of recombinant LIF protein for 24 hours when cells were confluent. (* indicates $p < 0.05$ compared to control within the same genotype, # indicates $p < 0.1$ compared to control within the same genotype, Δ indicates $p < 0.05$ compared to control of different genotype)

Discussion

Our investigation demonstrates that *ex vivo* transduction of immature hematopoietic progenitors with a LIF transgene and transplantation of those modified cells affect fibrosis in *mdx* mice. The findings show that our therapeutic approach can provide a translatable and targeted system of delivery that can ameliorate muscular dystrophy, which may also potentially be used for other diseases with a severe inflammatory component. Furthermore, our strategy offers distinct advantages over other current treatment strategies in which therapeutic cells or molecules are delivered systematically or are delivered to small areas of dystrophic muscle by injection. Our system provides temporally and spatially restricted delivery of therapeutic molecules specifically to sites of active inflammation throughout the body of the treated subject.

The primary treatment effect that we achieved in our investigation was a reduction of collagen type III expression in the diaphragm at early stages post-transplantation that was followed by reductions in collagen type III accumulation. Collagen type III, a fibrillar collagen that is thinner and less stiff than collagen type I, is normally found in high proportion in compliant tissues and early fetal tissues before its rate of synthesis is surpassed by that of collagen type I in adult tissue.[52,53] In the context of skeletal muscle, collagen type III synthesis increases rapidly in the early stages following injury.[54] As healing progresses, the ratio of intramuscular collagen type III to collagen type I returns to normal as collagen type III is broken down and replaced by collagen type I.[54] However, repetitive cycles of injury in dystrophic muscle accompanied by chronic inflammation can lead to dysregulation of collagen synthesis and breakdown, including collagen type III, that may contribute to fibrosis that can impair muscle regeneration and function.[13,55]

Despite significant decreases in the expression and accumulation of collagen type III in diaphragms of LV.LIF *mdx* mice, we observed no difference in collagen type I accumulation and only a trend for a reduction in its expression in *mdx* diaphragms. These dissimilar effects of LIF

on collagen types I and III differ from observations in other disease models. For example, LIF injection into kidneys in a rat model of renal fibrosis reduced mRNA and protein levels of both collagen types I and III, as measured by qPCR and Western blot.[56] Together, the findings show that although LIF is able to reduce expression of collagen types I and III *in vivo*, the effects of increasing LIF expression can vary with the specific pathology and tissue.

Our findings also show that the reduction in collagen type III accumulation in the LV.LIF *mdx* diaphragms was not accompanied by significant reductions on collagen type III in LV.LIF *mdx* quadriceps muscles. This negative finding may be attributable to a lack of sufficient statistical power caused by a small sample size. Given that neighboring areas of a muscle may be at different stages of pathology in DMD, the large size of the quadriceps muscles makes it more susceptible to error due to variability caused by asynchronous disease progression. Additionally, the discrepancy between treatment effects in the LV.LIF *mdx* diaphragms and quadriceps muscles may be due to differences in the fibrotic processes between *mdx* diaphragms and limb muscles; the *mdx* diaphragm experiences progressive fibrosis, whereas *mdx* limb muscles experience resolution after onset of pathology.[34,44-46] However, the underlying mechanisms that lead to their differences in fibrosis are not understood. Several factors may contribute to these mechanisms such as differences in inflammatory cells, expression of cytokines, and fibroblast and myogenic cell populations. For example, *mdx* diaphragms show higher mRNA levels of chemokine ligand 5 (CCL5) and macrophage inflammatory protein 1 alpha (MIP-1α), ligands for chemokine receptor 1 (CCR1), compared to *mdx* tibialis anterior (TA) muscles.[57] CCL5 and MIP-1α are chemokines involved in macrophage recruitment. Differences in their expression lead to differences in macrophage infiltration and polarization that can affect the fibrotic environment established by macrophage cytokine release.[58-61] Also, *mdx* diaphragm and quadriceps muscles express age-dependent differential mRNA levels of the TGF-β family of

profibrotic cytokines and their receptors.[62] Such differences in cytokine expression profiles can affect LIF's interactions with the tissue to regulate fibrosis.

We also tested whether LIF influences the number of HSP47+, collagen-producing cells in muscles to further explore the potential mechanisms underlying the muscle-specific effects we observed. However, we found that the number of HSP47+ cells did not change with LIF treatment in either diaphragm or quadriceps muscles. We also assayed whether the response to LIF stimulation of fibroblasts from diaphragms and quadriceps differed *in vitro*. We expected LIF stimulation to affect collagen type III expression in *mdx* diaphragm fibroblasts because collagen type III expression and accumulation in the diaphragms of LV.LIF *mdx* mice were reduced *in vivo.* However, *in vitro* stimulation with LIF caused a reduction in mRNA levels of collagen type III in fibroblasts from *mdx* quadriceps but not diaphragm muscles. LIF had no significant effect on collagen type I mRNA levels in either fibroblast populations. Together, these findings indicate that LIF may down-regulate collagen type III expression and accumulation *in vivo* by targeting a population of cells other than fibroblasts. In our previous studies, we showed that stimulation of myogenic progenitor cells with LIF *in vitro* decreased mRNA levels of collagen type III.[32] This finding points to myogenic progenitor cells as potential LIF targets that may play a primary role in the down-regulation of collagen type III expression *in vivo*.

The anti-fibrotic effect of LIF delivery to dystrophic muscle that we observed in the present study was much less effective than we achieved with other delivery approaches tested in our laboratory using LIF/*mdx* transgenic and LIF/*mdx* BMT mice. In the LIF/*mdx* transgenic mouse model, overexpression of a LIF transgene was driven by the CD11b promoter. LIF/*mdx* immature hematopoietic progenitors were transplanted into *mdx* mice to generate LIF/*mdx* BMT mice, in which donor-derived macrophages overexpress LIF. In the LIF/*mdx* transgenic mice, collagen type I accumulation was significantly reduced at one, three, and twelve months of age

in both diaphragm and TA muscles.[32] Collagen type III accumulation decreased at one and twelve months in the diaphragm, while no change was observed in the TA. These treatment effects accompanied functional improvements as indicated by reduced passive stiffness and higher mechanical efficiency with less energy dissipation during loading.[32] In the LIF/*mdx* BMT mouse model, both collagen type I and type III accumulation were reduced in the TA.[32]

Several factors may contribute to the difference in magnitude of treatment effects between the models. For example, all infiltrating CD11b+ cells in LIF/*mdx* mice can potentially overexpress LIF, but only donor-derived CD11b+ cells express the LIF transgene in LV.LIF *mdx* mice. In addition, resident macrophages overexpress LIF in LIF/*mdx* transgenic mice, which can provide a constant source of LIF for dystrophic muscle as they self-renew independent of infiltrating macrophages for their maintenance.[63] Thus, higher levels of LIF are delivered to dystrophic muscle in LIF/*mdx* than in LV.LIF *mdx* mice, which may cause greater treatment effects.

The differences in treatment outcomes in our current experiment compared to the LIF/*mdx* BMT model may also partially result from differences in the treatment of mice prior to transplantation.[32] In our current model, we used whole body irradiation to ablate the immune system, while the LIF/*mdx* BMT model employed myeloablative preconditioning via 1,4-butanediol dimethanesulfonate (busulfan).[32] The LV.LIF *mdx* mice showed high variability in chimeric levels with a mean of 65.6%. On the other hand, the mean chimeric level for the busulfan-treated LIF/*mdx* BMT mice was 87%.[32] Busulfan has been shown to yield chimeric levels greater than 80% in mice, and donor cells survive for at least one year after transplantation.[64] This suggests that busulfan may be more effective in ensuring successful engraftment of the transplanted immature hematopoietic progenitors in the recipient mice. Additionally, irradiation leads to persistent elevated expression of proinflammatory cytokines such as IL-1α and TNFα and can modulate macrophage polarization and activity.[65-68] Busulfan,

on the contrary, does not promote persistent elevated proinflammatory cytokine expression.[69] Thus, busulfan may be a more effective myeloablative preconditioning treatment for an approach involving macrophages as a therapeutic vehicle to avoid alterations in proinflammatory cytokine expression and ensure complete ablation of hematopoietic progenitors and successful engraftment of donor cells.

The ages at which transplantation occurred in the two models is another important difference in the two investigations. Despite allowing more time between transplantation and analysis, LV.LIF *mdx* mice exhibited smaller treatment effects compared to LIF/*mdx* BMT mice. This difference may be attributed to the age at which transplantation occurred. *Mdx* mice experience acute onset of dystrophic pathology at three to four weeks of age.[33,44,46] LIF/*mdx* BMT mice underwent transplantation at two-months-old, whereas LV.LIF *mdx* mice underwent transplantation at three- to four-months-old. Stronger treatment effects observed in LIF/*mdx* BMT mice suggest that transplantation at an age closer to the onset of pathology may allow LIF to more effectively reduce fibrosis.

Altogether, our findings show that CD11b-driven overexpression of LIF by macrophages via *ex vivo* transduction and transplantation of immature hematopoietic progenitors can affect muscle fibrosis in *mdx* mice. Furthermore, we demonstrate that macrophage delivery of a therapeutic transgene can become more translatable by *ex vivo* transduction and transplantation of modified immature hematopoietic progenitors. However, our findings also show that our novel approach requires additional investigation to generate stronger treatment effects. For example, our delivery system needs to be tested with other myeloablative preconditioning treatments, particularly busulfan, to find a regimen that will ensure the best chances of engraftment. Performing the transplantation at an earlier time may also allow LIF to further slow the rate of progression of fibrosis. Notably, our approach differentially reduced fibrosis but did not affect

muscle regeneration or inflammation. The lack of treatment effects on these disease components indicate that a different therapeutic agent may be more useful in our approach. Testing our delivery system with another transgene will allow greater understanding of the effectiveness of our approach and LIF as a therapeutic agent. Overall, our delivery system paves the way for macrophages as a translatable therapeutic approach to ameliorate muscular dystrophy and other diseases with an inflammatory component.

References

1. Hoffman, E.P., Brown, R.H.Jr., Kunkel L.M. (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell, 51, 919-928.
2. Petrof B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M., Sweeney, H.L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proceedings of the National Academy of Sciences of the United States of America, 90, 3710-3714.
3. Jennekens, F.G., Ten Tate, L.P., De Visser, M., Wintzen, A.R. (1991) Diagnostic criteria for Duchenne and Becker muscular dystrophy and myotonic dystrophy. Neuromuscular Disorders 1(6): 389-391.
4. De Bruin, P.F., Ueki, J., Bush, A., Khan, Y., Watson, A., Pride, N.B. (1997) Diaphragm thickness and respiratory strength in patients with Duchenne muscular dystrophy. Thorax 52(5): 473-475.
5. Brigitte, M. Schilte, C., Plonquet, A., Baba-Amer, Y., Henri, A., Charlier, C., Tajbakhsh, S., Albert, M., Gherardi, R.K., Chrétien, F. (2010) Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury. Arthritis and Rheumatism 62(1): 268-279.
6. Fielding, R.A., Manfredi, T.J., Ding, W., Fiatarone, M.A., Evans, W.J., Cannon, J.G. (1993) Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle. The American Journal of Physiology 265(1 Pt 2): R166-172.
7. Pace, J.L., Russell, S.W., Schreiber, R.D., Altman, A., Katz, D.H. (1983) Macrophage activation: priming activity from a T-cell hybridoma is attributable to interferon-gamma. Proceedings of the National Academy of Sciences of the United States of America 80(12): 3782-2786.

8. Philip, R. and Epstein, L.B. (1986) Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature 323(6083): 86-89.
9. Villalta, S.A., Nguyen, H.X., Deng, B., Gotoh, T., Tidball, J.G. (2009) Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Human Molecular Genetics 8(3): 482-496.
10. Stein, M., Keshav, S., Harris, N., Gordon, S. (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. The Journal of Experimental Medicine 176(1): 287-292.
11. Bell, C.D. and Conen, P.E. (1968) Histopathological changes in Duchenne muscular dystrophy. Journal of the Neurological Sciences 7(3): 529-544.
12. Tidball, J.G., Dorshkind, K., Wehling-Henricks, M. (2014) Shared signaling systems in myeloid cell-mediated muscle regeneration. Development 141(6): 1184-1196.
13. Serrano, A.L. and Muñoz-Cánoves, P. (2010) Regulation and dysregulation of fibrosis in skeletal muscle. Experimental Cell Research 316(18): 3050-3058.
14. Tidball, J.G. and Wehling-Henricks, M. (2007) Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice *in vivo*. The Journal of Physiology 578(Pt 1): 327-336.
15. Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R.K., Chazaud, B. (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. Journal of Experimental Medicine 204(5): 1057-1069.
16. Bencze, M., Negroni, E., Vallese, D., Yacoub-Youssef, H., Chaouch, S., Wolff, A., Aamiri, A., Di Santo, J.P., Chazaud, B., Butler-Browne, G., Savino, W., Mouly, V., Riederer, I. (2012) Proinflammatory macrophages enhance the regenerative capacity of

human myoblasts by modifying their kinetics of proliferation and differentiation. Molecular Therapy 20(11): 2168-2179.

17. Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J., Hill, A.M. (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. Journal of Immunology 164(12) 6166-6173.
18. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M. (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends in Immunology 25(12): 677-686.
19. de Waal Malefyt, R., Figdor, C.G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G., de Vries, J.E. (1993) Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-gamma or IL-10. Journal of Immunology 151(11): 6370-6381.
20. Wehling-Henricks, M., Jordan, M.C., Gotoh, T., Grody, W.W., Roos, K.P., Tidball, J.G. (2010) Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in *mdx* muscular dystrophy. Public Library of Science ONE 5(5): e10763.
21. Tidball, J.G. and Villalta, S.A. (2010) Regulatory interactions between muscle and the immune system during muscle regeneration. American Journal of Physiology Regulatory, Integrative, and Comparative Physiology 298(5): R1173-1187.
22. Yue, Y., Yang, X., Feng, K., Wang, L., Hou, J., Mei, B., Qin, H., Liang, M., Chen, G., Wu, Z. (2017) M2b macrophages reduce early reperfusion injury after myocardial ischemia in mice: A predominant role of inhibiting apoptosis via A20. International Journal of Cardiology 245:228-235.
23. Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., Muñoz-Cánoves, P. (2011) Aberrant repair and fibrosis development in skeletal muscle. Skeletal Muscle 1: 21.

24. Wehling, M., Spencer, M.J., Tidball, J.G. (2001) A nitric oxide synthase transgene ameliorates muscular dystrophy in *mdx* mice. Journal of Cell Biology 155(1): 123-132.
25. Manzur, A.Y., Kuntzer, T., Pike, M., Swan, A. (2008) Glucocorticoid corticosteroids for Duchenne muscular dystrophy. The Cochrane Database of Systematic Reviews (1): CD003725.
26. Beytía Mde L., Vry, J., Kirschner, J. (2012) Drug treatment of Duchenne muscular dystrophy: available evidence and perspectives. Acta Myologica 31(1): 4-8.
27. Reining, A.M., Mirzaei, S., Berlau, D.J. (2017) Advances in the treatment of Duchenne muscular dystrophy: new and emerging pharmacotherapies. Pharmacotherapy 37(4): 492-299.
28. Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R., Nabeshima, Y.I. (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390(6655):45-51.
29. Wehling-Henricks, M., Li, Z., Lindsey, C., Wang, Y., Welc, S.S., Ramos, J.N., Khanlou, N., Kuro-O, M., Tidball, J.G. (2016) Klotho gene silencing promotes pathology in the *mdx* mouse model of Duchenne muscular dystrophy. Human Molecular Genetics 25(12):2465-2482.
30. Wehling-Henricks, M., Welc, S.S., Samengo, G., Rinaldi, C., Lindsey, C., Wang, Y., Lee, J., Kuro-O, M., Tidball, J.G. (2018) Macrophages escape Klotho gene silencing in the *mdx* mouse model of Duchenne muscular dystrophy and promote muscle growth and increase satellite cell numbers through a Klotho-mediated pathway. Human Molecular Genetics 27(1):14-29.
31. Nicola, N.A., Babon, J.J., (2015) Leukemia inhibitory factor (LIF). Cytokine and Growth Factor Reviews 26(5): 533-544.

32. Welc, S.S., Flores, I., Wehling-Henricks, M., Ramos, J., Wang, Y., Bertoni, C., Tidball, J.G. (2019) Targeting a therapeutic LIF transgene to muscle via the immune system ameliorates muscular dystrophy. Nature Communications 10(1): 2788.
33. Bulfield, G., Siller, W.G., Wight, P.A.L., Moore, K.J. (1984) X chromosome-linked muscular dystrophy (*mdx)* in the mouse. Proceedings of the National Academy of Sciences of the United States of America 81(4):1189-92.
34. Stedman, H.H., Sweeney, H.L., Shrager, J.B., Maguire, H.C., Panettieri, H.A., Petrof, B., Narusawa, M., Leferovich, J.M., Sladky, J.T., Kelly, A.M. (1991) The *mdx* mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. Nature 352(6335):536-539.
35. Barnard, W., Bower, J., Brown, M.A., Murphy, M., Austin, L. (1994) Leukemia inhibitory factor (LIF) infusion stimulates skeletal muscle regeneration after injury: injured muscle expresses lif mRNA. Journal of the Neurological Sciences 123(1-2): 108-113.
36. Kurek, J., Bower, J., Romanella, M., Austin, L. (1996) Leukemia inhibitory factor treatment stimulates muscle regeneration in the *mdx* mouse. Neuroscience Letters 212(3):167-170.
37. Austin, L., Bower, J.J., Bennett, T.M., Lynch, G.S., Kapsa, R., White, J.D., Barnard, W., Gregorevic, P., Byrne, E. (2000) Leukemia inhibitory factor ameliorates muscle fiber degeneration in the *mdx* mouse. Muscle & Nerve 23(11): 1700-1705.
38. Austin, L. and Burgess, A.W. (1991) Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. Journal of the Neurological Sciences 101(2): 193-197.
39. Austin, L., Bower, J., Kurek, J., Vakakis, N. (1992) Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. Journal of the Neurological Sciences 112(1-2): 185-191.

40. Hunt, L., Tudor, E., White, J. (2010) Leukemia inhibitory factor-dependent increase in myoblast cell number is associated with phosphotidylinositol 3-kinase-mediated inhibition of apoptosis and not mitosis. Experimental Cell Research 316(6):1002-1009.
41. Todd, R.F., Nadler, L.M., Schlossman, S.F. (1981) Antigens on human monocytes identified by monoclonal antibodies. The Journal of Immunology 126(4): 1435-1442.
42. Rosmarin, A.G., Weil, S.C., Rosner, G.L., Griffin, J.D., Arnaout, M.A., Tenen, D.G. (1989) Differential expression of Cd11b/CD18 (Mo1) and myeloperoxidase genes during myeloid differentiation. Blood 73(1): 131-136.
43. Dziennis, S., Van Etten, R.A., Pahl, H.L., Morris, D.L., Rothsein, T.L., Blosch, C.M., Perlmutter, R.M., and Tenen, D.G. (1995) The CD11b promoter directs high-level expression of reporter genes in macrophages in transgenic mice. Blood 85(2): 319-329.
44. Dangain, J. and Vrbova, G. (1984) Muscle development in *mdx* mutant mice. Muscle & Nerve 7(9):700-704.
45. Carnwath, J.W. and Shotton, D.M. (1987) Muscular dystrophy in the *mdx* mouse: histopathology of the soleus and extensor digitorum longus muscles. Journal of the Neurological Sciences 80(1):39-54.
46. DiMario, J.X., Uzman, A., Strohman, R.C. (1991) Fiber regeneration is not persistent in dystrophic (*mdx*) mouse skeletal muscle. Developmental Biology 148(1): 314-321.
47. Ito, S. and Nagata, K. (2017) Biology of Hsp47 (Serpin H1), a collagen-specific molecular chaperone. Seminars in Cell & Developmental Biology 62:142-151.
48. Page-McCaw, A., Ewald, A.J., Werb, Z. (2007) Matrix metalloproteinases and the regulation of tissue remodeling. Nature Reviews Molecular Cell Biology 8(3): 221–233.
49. Fukushima, K., Nakamura, A., Ueda, H., Yuasa, K., Yoshida, K., Takeda, S., Ikeda, S. (2007) Activation and localization of matrix metalloproteinase-2 and -9 in the skeletal muscle of the muscular dystrophy dog (CXMDJ). BMC Musculoskeletal Disorders 8:54.

50. Arpino, V., Brock, M., Gill, S.E. (2015) The role of TIMPs in regulation of extracellular matrix proteolysis. Matrix Biology 44-46:247-254.

51. Wang, F., Trial, J., Diwan, A., Gao, F., Birdsall, H., Entman, M., Hornsby, P., Sivasubramaniam, N., Mann, D. (2002) Regulation of cardiac fibroblast cellular function by leukemia inhibitory factor. Journal of Molecular and Cellular Cardiology 34(10): 1309-1316.

52. Gelse, K., Pöschl, E., Aigner, T. (2003) Collagens—structure, function, and biosynthesis. Advanced Drug Delivery Reviews 55(12): 1531-1546.

53. Epstein, E.H., Jr. (1974) $[\alpha 1(III)]_3$ human skin collagen. The Journal of Biological Chemistry 249(10) 3225-3231.

54. Lehto, M., Sims, T.J., Bailey, A.J. (1985) Skeletal muscle injury—molecular changes in the collagen during healing. Research in Experimental Medicine 185(2): 95-106.

55. Duance, V.C., Stephens, H.R., Dunn, M., Bailey, A.J., Dubowitz, V. (1980) A role for collagen in the pathogenesis of muscular dystrophy? Nature 284: 470-472.

56. Yu, Y., Wang, Y., Niu, Y., Fu, L., Chin, Y.E., Yu, C. (2015) Leukemia inhibitory factor attenuates renal fibrosis through Stat3-miR-29c. American Journal of Physiology-Renal Physiology 309(7): F595-603.

57. Demoule, A., Divangahi, M., Danialou, G., Gvozdic, D., Larkin, G., Weishang, B., Petrof, B.J. (2005) Expression and regulation of CC class chemokines in the dystrophic (*mdx*) diaphragm. American Journal of Respiratory Cell and Molecular Biology 33(2):178-185.

58. DiPietro, L.A., Burdick, M., Low, Q.E., Kunkei, S.L., Strieter, R.M. (1998) MIP-1α as a critical macrophage chemoattractant in murine wound repair. The Journal of Clinical Investigation 101(8): 1693-1698.

59. Porter, J.D., Guo, W., Merriam, A.P., Khanna, S., Cheng, G., Zhou, X., Andrade, F.H., Richmonds, C., Kaminski, H.J. (2003) Persistent over-expression of specific CC class

chemokines correlates with macrophage and T-cell recruitment in *mdx* skeletal muscle. Neuromuscular Disorders 13(3): 223-235.

60. Li, M., Sun, X., Zhao, J., Xia, L., Li, J., Xu, M., Wang, B., Guo, H., Yu, C., Gao, Y., Wu, H., Kong, X., Xia, Q. (2019) CCL5 deficiency promotes liver repair by improving inflammation resolution and liver regeneration through M2 macrophage polarization. Cellular & Molecular Immunology 1-12.
61. Berres, M-L., Koenen, R.R., Rueland, A., Zaldivar, M.M., Heinrichs, D., Sahin, H., Schmitz, P., Streetz, K.L., Berg, T., Gassler, N., Weiskirchen, R., Proudfoot, A., Weber, C., Trautweiv, C., Wasmuth, H.E. (2010) Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. The Journal of Clinical Investigation 120(11): 4129-4140.
62. Zhou, L.., Porter, J.D., Cheng, G., Gong, B., Hatala, D.A., Merriam, A.P., Zhou, X., Rafael, J..A. Kaminski, H.J. (2006) Temporal and spatial mRNA expression patterns of TGF-β1, 2, 3 and TβRI, II, III in skeletal muscles of *mdx* mice. Neuromuscular Disorders 16(1): 32-38.
63. Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M.B., Lebeouf, M., Becker, C.D., See, P., Price, J., Lucas, D., Greter, M., Mortha, A., Boyer, S.W., Forsberg, E.C., Tanaka, M., van Rooijen, N., Garcia-Sastre, A., Stanley, E.R., Merad, M. (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity 38(4): 792-804.
64. Peake, K., Manning, J., Lewis, C.-A., Barr, C., Rossi, F., Krieger, C. (2015) Busulfan as a myelosuppressive agent for generating stable high-level bone marrow chimerism in mice. Journal of Visualized Experiments (98): 52553.
65. Rubin, P., Johnston, C.J., Williams, J.P., McDonald, S., Finkelstein, J.N. (1995) A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. International Journal of Radiation Oncology, Biology, Physics 33(1): 99-109.

66. Johnston, C.J., Piedboeuf, B., Rubin, P., Williams, J.P., Baggs, R., Finkelstein, J.N. (1996) Early and persistent alterations in expression of interleukin-1α, interleukin-1β and tumor necrosis factor α mRNA levels in fibrosis-resistant and sensitive mice after thoracic irradiation. Radiation Research 145(6): 762-767.
67. Teresa Pinto, A., Laranjeiro Pinto, M., Patricia Cardoso, A., Monteiro, C., Teixeira Pinto, M., Filipe Maia, A., Castro, P., Figueira, R., Monteiro, A., Marques, M., Mareel, M., Dos Santos, S.G., Seruca, R., Adolfo Barbosa, M., Rocha, S., José Oliveira, M. (2016) Ioninzing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities. Scientific Reports 6: 18765.
68. Ibuki, Y., Mizuno, S., Goto, R. (2003) γ-irradiation-induced DNA damage enhances NO production via NF-κB activation in RAW264.7 cells. Biochimica et Biophysica Acta 1593(2-3): 159-167.
69. Wilkinson, F.L., Sergijenko, A., Langford-Smith, K.J., Malinowska, M., Wynn, R.F., Bigger, B.W. (2013) Busulfan conditioning enhances engraftment of hematopoietic donor-derived cells in the brain compared with irradiation. Molecular Therapy 21(4): 868-876.