

VEGF induces stress fiber formation in fibroblasts isolated from dystrophic muscle

Kelly M. Gutpell^{1,2,3} · Lisa M. Hoffman^{1,2,3,4}

Received: 31 March 2015 / Accepted: 16 July 2015 / Published online: 29 July 2015
© The International CCN Society 2015

Abstract Treatment with vascular endothelial growth factor (VEGF) to reduce ischemia and enhance both endogenous muscle repair and regenerative cell therapy in Duchenne muscular dystrophy (DMD) has been widely proposed in recent years. However, the interaction between angiogenesis and fibrosis, a hallmark feature of DMD, remains unclear. To date, it has not been determined whether VEGF exerts a pro-fibrotic effect on DMD-derived fibroblasts, which may contribute to further disease progression. Thus, the purpose of this study was to investigate the effect of exogenous VEGF on fibroblast cultures established from a murine model of DMD. Primary fibroblast cultures were established from gastrocnemius and diaphragm muscles of 10 week-old mdx/utrn^{+/-} mice. Quantitative polymerase chain reaction (qPCR) was employed to assess changes in transcript expression of alpha-smooth muscle actin (*Acta2*), type-1 collagen (*Colla1*), connective tissue growth factor (*Ctgf/ccn2*) and fibronectin (*Fnl*). Immunofluorescence and Western blot analysis was further employed to visualize changes in protein expression of alpha-smooth muscle actin (α -SMA), CTGF/CCN2 and fibronectin. mRNA levels of *Colla1*, *Ctgf/ccn2*, and *FN* did not increase

following treatment with VEGF in fibroblasts derived from either diaphragm or gastrocnemius muscles. *Acta2* expression increased significantly in diaphragm-derived fibroblasts following treatment with VEGF. Morphological assessment revealed increased stress fiber formation in VEGF-treated fibroblasts compared to the untreated control fibroblasts. The findings from this study suggest that further investigation into the effect of VEGF on fibroblast function is required prior to the utilization of the growth factor as a treatment for DMD.

Keywords Angiogenesis · Duchenne muscular dystrophy · Dystrophin · Fibrosis · Skeletal muscle · Vascular endothelial growth factor

Abbreviations

DMD	Duchenne muscular dystrophy
VEGF	Vascular endothelial growth factor
TGF β	Transforming growth factor beta
<i>Colla1</i>	Type I collagen (mRNA)
α SMA	Alpha-smooth muscle actin
<i>Acta2</i>	Alpha-smooth muscle actin (mRNA)
<i>Ctgf/ccn2</i>	Connective tissue growth factor (mRNA)
CTGF/CCN2	Connective tissue growth factor
<i>Fnl</i>	Fibronectin (mRNA)
FN	Fibronectin
qPCR	Quantitative polymerase chain reaction

✉ Lisa M. Hoffman
lhoffman@lawsonimaging.ca
Kelly M. Gutpell
kgutpell@uwo.ca

- ¹ Lawson Health Research Institute, London, Ontario, Canada
- ² Department of Anatomy and Cell Biology, Western University, London, Ontario, Canada
- ³ Department of Medical Biophysics, Western University, London, Ontario, Canada
- ⁴ Imaging Program, Lawson Health Research Institute, 268 Grosvenor Street, London, Ontario, Canada N6A 4V2

Introduction

Angiogenic therapy has been proposed as a potential therapeutic agent for treating skeletal muscle ischemia inherent to Duchenne muscular dystrophy (DMD) (Shimizu-Motohashi and Asakura 2014). To this end, a number of studies have

now used a potent inducer of angiogenesis, vascular endothelial growth factor (VEGF), as a therapeutic agent in the mdx mouse (Messina et al. 2007; Gregorevic et al. 2004; Beckman et al. 2013). Importantly, enhanced vascularization in dystrophic muscle has been shown to enhance the efficacy of regeneration through cell therapy (Bouchentouf et al. 2008). One aspect of these studies that has not been directly addressed is the effect of exogenous VEGF on skeletal muscle fibroblasts. Indeed, recent studies indicate that high doses of VEGF induce fibrosis in inflammatory and non-inflammatory stages of systemic scleroderma (SSc) (Maurer et al. 2014). Similarly, fibroblasts isolated from patients with idiopathic pulmonary fibrosis exhibit a decreased fibrotic response following treatment with a VEGF receptor blocker, nintedanib (Hostettler et al. 2014). The findings in the SSc study are particularly important because they highlight the differential effect observed with low levels of circulating VEGF versus high doses of VEGF typically associated with exogenous treatment. In contrast, studies utilizing murine models of DMD have shown that a loss of endogenous VEGF may have deleterious effects on regeneration. Additionally, results from studies that have administered VEGF to dystrophic muscle have suggested an anti-fibrotic role of the growth factor in this disease. While the latter findings highlight the immense therapeutic potential of VEGF to treat DMD, there is currently a lack of studies that directly investigate the effect of exogenous VEGF treatment on progression of fibrosis (Miyazaki et al. 2011; Rhoads et al. 2013).

Fibrosis develops in DMD patients and is largely responsible for disease fatality (Desguerre et al. 2009; Chen et al. 2005; Bernasconi et al. 1995). Since the mdx mouse model does not develop fibrosis to the same extent as is observed in patients, studying the effect of VEGF on the fibrotic response in this model may lead to an underestimation of its effect on fibrosis (Desguerre et al. 2012; Zhou et al. 2008). More recent studies have used a double knockout mutant that lacks both dystrophin and utrophin (dko), but this mouse is severely affected and does not live past 20 weeks of age (Deconinck et al. 1997; Goyenvalle et al. 2010, 2012; van Putten et al. 2012). We and others have demonstrated that the heterozygous mdx/utrn^{+/-} mouse develops hind limb skeletal muscle fibrosis earlier in life than its mdx counterpart, but maintains a longer lifespan of approximately 2 years in laboratory conditions (Zhou et al. 2008; Gutpell et al. 2015). Isolation of primary fibroblasts from this mouse model thus provides a means to directly assess the effect of exogenous VEGF treatment on the expression of pro-fibrotic genes (Mezzano et al. 2007). Another important consideration for angiogenic therapy is the effect of treatment on different muscles affected by DMD. Indeed, all three murine models of DMD—mdx, mdx/utrn^{+/-} and dko—develop fibrosis in the diaphragm muscle, and to a greater extent than what is observed in the hind limb musculature of any of these mice (Stedman et al. 1991; Huang et al.

2011). The underlying mechanism for this differential progression of fibrosis has not been thoroughly investigated. Therefore, this study also investigated whether the effect of VEGF treatment differs in diaphragm-derived fibroblasts versus gastrocnemius-derived fibroblasts.

Materials and methods

Animal care and ethics statement

All experiments were performed at Lawson Health Research Institute at St. Joseph's Health Care (SJHC) in London, Ontario. The mdx/utrn^{+/-} mice, originally generated by Dr.'s Mark Grady and Josh Sanes (Washington University, St. Louis) (Grady et al. 1997a, b), were generously provided by Dr. Robert Grange (Virginia Polytechnic and State University) and maintained in the Animal Care Facility at SJHC. Colonies were maintained under controlled conditions (19–23 °C, 12 h light/dark cycles) and allowed water and food ad libitum. Only 10 week-old mdx/utrn^{+/-} mice were used in this study. All procedures involving animal experiments were carried out in strict accordance with the Canadian Council on Animal Care (CCAC) and were approved by the Animal Use Subcommittee at Western University.

Primary fibroblast isolation

Gastrocnemius and diaphragm tissue samples were isolated from 10-week old mdx/utrn^{+/-} mice. The isolation protocol was adapted from a method previously described by others (Mezzano et al. 2007). Briefly, mice were sacrificed via gas euthanasia and dissected muscles were placed in sterile phosphate-buffered saline (PBS) and transferred to a cell culture hood. Tissues were minced into 1–2 mm³ pieces and placed on a gelatin-coated 10 cm dish with sterile tweezers. Approximately 20–25 pieces were plated per dish. The tissue was then covered with primary fibroblast isolation medium (DMEM/F12 supplemented with 20 % FBS plus penicillin and streptomycin) and incubated at 37 °C. By day 4, fibroblasts began exiting the tissue explants. Fibroblast medium (DMEM/F12 supplemented with 20 % FBS and penicillin and streptomycin) was changed every other day from this point. Cells were passaged at day 7 using 0.25 % trypsin-EDTA. Tissue explants were removed by running cells through a 100 µm nylon filter. Only cells in passages 2–4 were used for experimental purposes.

Growth factor supplementation

1×10^5 fibroblasts were seeded per well onto a 12-well plate and incubated overnight. The next day, cells were serum-deprived in reduced-serum fibroblast medium (DMEM/F12

5 % FBS plus penicillin and streptomycin) for 6 h. Following serum deprivation, cells were treated with 50 ng/ml recombinant mouse VEGF₁₆₄ (R&D Systems), 50 ng/ml transforming growth factor- β (TGF β), or untreated. Growth factor doses were determined from previous pilot studies in our lab. TGF β treatment served as a positive control for inducing a fibrotic response and the untreated fibroblasts served as a negative control. Cells were harvested 6 and 18 h later for subsequent RNA extraction and 48 h later for protein extraction.

Quantitative real-time polymerase chain reaction

RNA was extracted using a QIAGEN RNeasy Plus Mini Kit. RNA concentration and quality was assessed using a Nanodrop Spectrophotometer ND-1000. 1–2 μ g of high-quality RNA was reverse-transcribed using the High-Capacity RNA-to-cDNA Kit (Life Technologies). Following primer validation, Taqman Gene Expression Assays were used to measure *Ctgf/ccn2* (Mm01192932_g1), *Coll1a1* (Mm00801666_g1), *Acta2* (Mm01546133_m1) and *Fnl1* (Mm01256744_m1) mRNA expression relative to *Gapdh* endogenous control (Mm99999915_g1) levels using the $\Delta\Delta$ CT method on a Real-Time PCR Applied Biosystems Inc. Prism 7500 under the following conditions: Initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation (95 °C for 15 s), primer annealing (60 °C for 1 min) and transcript extension (50 °C for 2 min). All samples were run in triplicate.

Immunocytochemistry

Fibroblasts were processed for immunocytochemistry by fixing in 2–4 % paraformaldehyde for 20 min. Fibroblasts were then washed three times with phosphate-buffered saline (PBS), and incubated at room temperature in blocking buffer (1 % BSA, 10 % goat serum in PBS) for 45 min. Fibroblasts were incubated with anti- α -SMA (Abcam, 1:100) primary antibody. Following thorough washing with PBS, Alexafluor 488-IgG (Life Technologies, 1:1000) secondary antibody was used to visualize the primary antibody, and ProLong Gold anti-fade with DAPI (Life Technologies) was added to visualize the nuclei and to mount the coverslips onto glass slides. Fluorescent images were acquired at a twenty times magnification on a Nikon Eclipse microscope.

Western blot

Cells were collected in Phosphosafe lysis buffer containing a protease inhibitor cocktail and lysed via sonication. Cell culture supernatants were collected and lyophilized for 210 min at 45 °C and re-suspended in PBS. Protein was quantified using the bicinchoninic acid assay (Pierce). Fifty microgram of protein was heat denatured and loaded onto a TGX stain-

free SDS gel. Protein was transferred onto a PVDF low fluorescence membrane using the Transblot Turbo machine (Bio-Rad) and total protein was visualized on the Bio-Rad Gel Doc system. Membranes were blocked with 5 % bovine serum albumin in tris-buffered saline containing 0.05 % tween 20 for 1 h. Blots were then incubated with primary anti-fibronectin antibody (1:1000, Abcam) at 4 °C overnight. Following thorough washing in TBS-T, blots were incubated with anti-rabbit horse radish peroxidase secondary antibody (1:5000) for 1 h. Bands were visualized via chemiluminescence using ImageLab software (Bio-Rad) and normalized to total protein signal from the stain-free blots (Taylor et al. 2013). Bands from cell culture supernatants are shown for qualitative purposes only since normalization to total protein was not possible given the signal saturation from the serum.

Statistical analysis

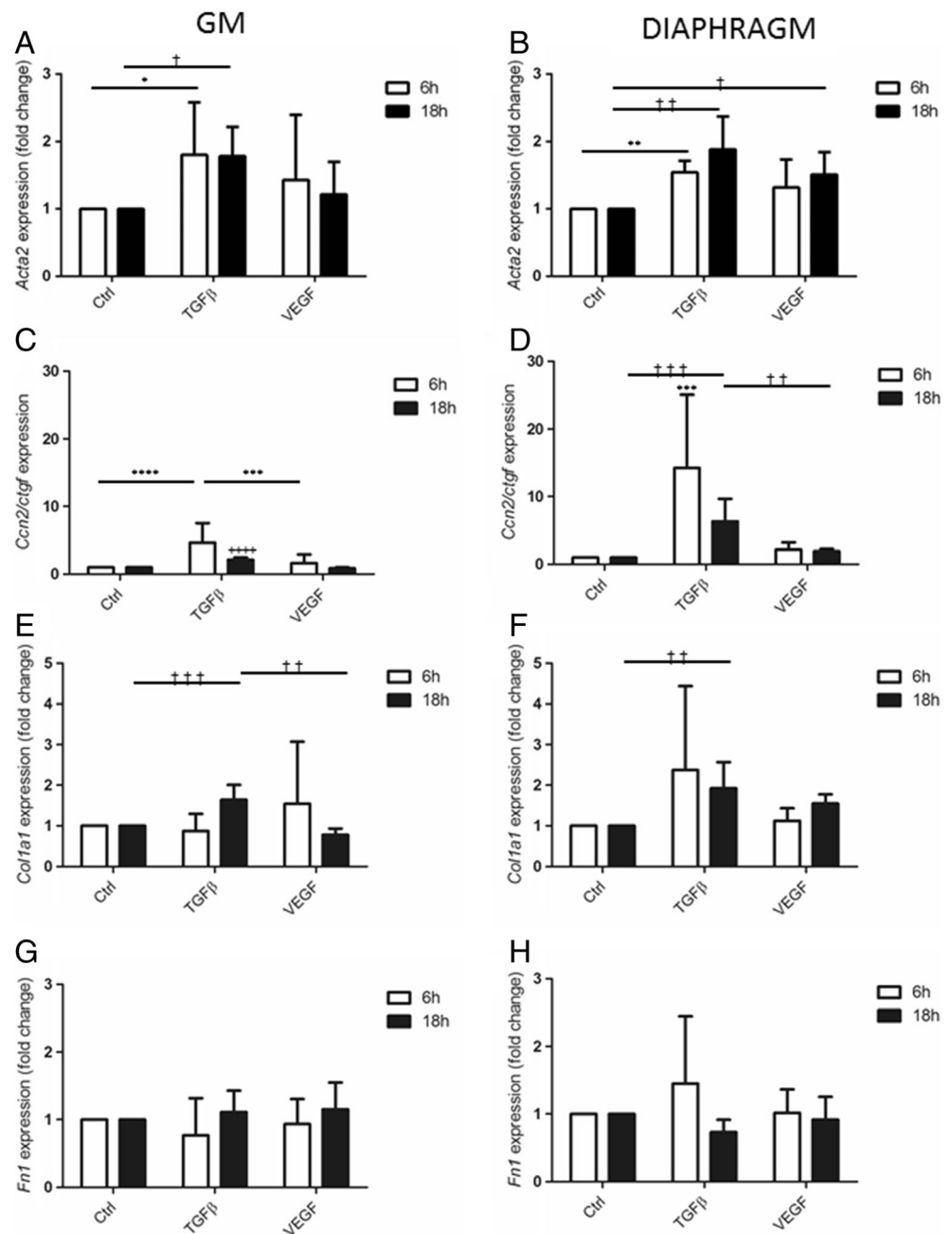
Gene expression data was analyzed using a two-way analysis of variance (ANOVA) to determine simple main effects of treatment and time point followed by individual one-way ANOVA to assess differences within the 6 and 18 h time points. Differences between groups were determined using Tukey's post-hoc test. A *p*-value less than 0.05 was considered significant (*n*=6 and *n*=4 for gene expression and protein experiments, respectively).

Results

Effect of VEGF on fibrotic gene expression

Expression of multiple genes involved in dysregulated tissue repair was measured to investigate whether VEGF induces a fibrotic response in fibroblasts derived from dystrophic muscle (Fig. 1). Specifically, changes in mRNA levels of alpha-smooth muscle actin (*Acta2*), CTGF/CCN2 (*Ctgf/ccn2*), type I collagen (*Coll1a1*) and fibronectin (*Fnl1*) were assessed in diaphragm and gastrocnemius (GM)-derived fibroblasts. *Acta2* expression significantly increased in GM-derived fibroblasts 1.8-fold following TGF β treatment, but not following VEGF treatment (Fig. 1a). *Acta2* expression in diaphragm-derived fibroblasts, on the other hand, responded to both TGF β treatment as well as VEGF treatment (Fig. 1b). TGF β induced a 1.5-fold increase compared to control cells as early as 6 h after administration of the growth factor. By 18 h, *Acta2* expression was 1.9- and 1.5-fold higher in fibroblasts treated with TGF β and VEGF, respectively (*p*<0.0001). TGF β increased expression of *Ctgf/ccn2* in both GM- and diaphragm-derived fibroblasts. Six hours following administration, a 4.6- and 14.2-fold increase in expression of the gene was measured in GM (*p*<0.0001) and diaphragm fibroblasts (*p*=0.0002), respectively (Fig. 1c and d). This level of *Ctgf/ccn2* expression

Fig. 1 Effect of VEGF on expression of genes involved in dysregulated tissue repair in fibroblasts derived from GM or diaphragm muscle of mdx/utrn^{-/-} mice. Fibroblasts isolated from gastrocnemius (a, c, e, g) or diaphragm muscles (b, d, f, h) of mdx/utrn^{-/-} mice were treated with VEGF₁₆₄ for 6 or 18 h. Treatment with TGFβ, a known-inducer of pro-fibrotic gene expression, was used as a positive control. *Acta2* (a, b), *Ctgf/ccn2* (c, d), *Coll1a1* (e, f) and *Fn1* (g, h) expression levels were measured relative to *Gapdh* ($n=6$, *significant difference between treatment groups within the 6 h time point, †significant difference between groups within the 18 h time point. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001\pm$ SD)



was significantly higher than expression in either control fibroblasts or VEGF-treated fibroblasts. There was no measurable increase in *Ctgf/ccn2* transcript level following VEGF treatment in either diaphragm or GM fibroblasts.

To determine if VEGF stimulates expression of genes that encode extracellular matrix proteins, we assessed changes in mRNA expression of fibronectin and type I collagen. Neither TGFβ nor VEGF altered expression of either *Fn1* or *Coll1a1* by any time points assessed in this study (Fig. 1e–h). Conversely, Western blot analysis of fibronectin protein (FN) confirmed that VEGF, but not TGFβ, increased expression of the protein by 1.9-fold in diaphragm fibroblasts ($p=0.029$) and by

1.5-fold in GM fibroblasts ($p=0.001$). Similar qualitative trends were observed in FN levels in the supernatant (Fig. 2).

Qualitative changes in αSMA expression following VEGF treatment

Immunocytochemical analysis revealed minimal αSMA expression in both GM and diaphragm fibroblasts. While all αSMA protein expressed in GM-fibroblasts was located in the cytoplasm, some αSMA-positive diaphragm fibroblasts expressed the protein within stress fibers. TGFβ induced a robust response in diaphragm fibroblasts resulting in high

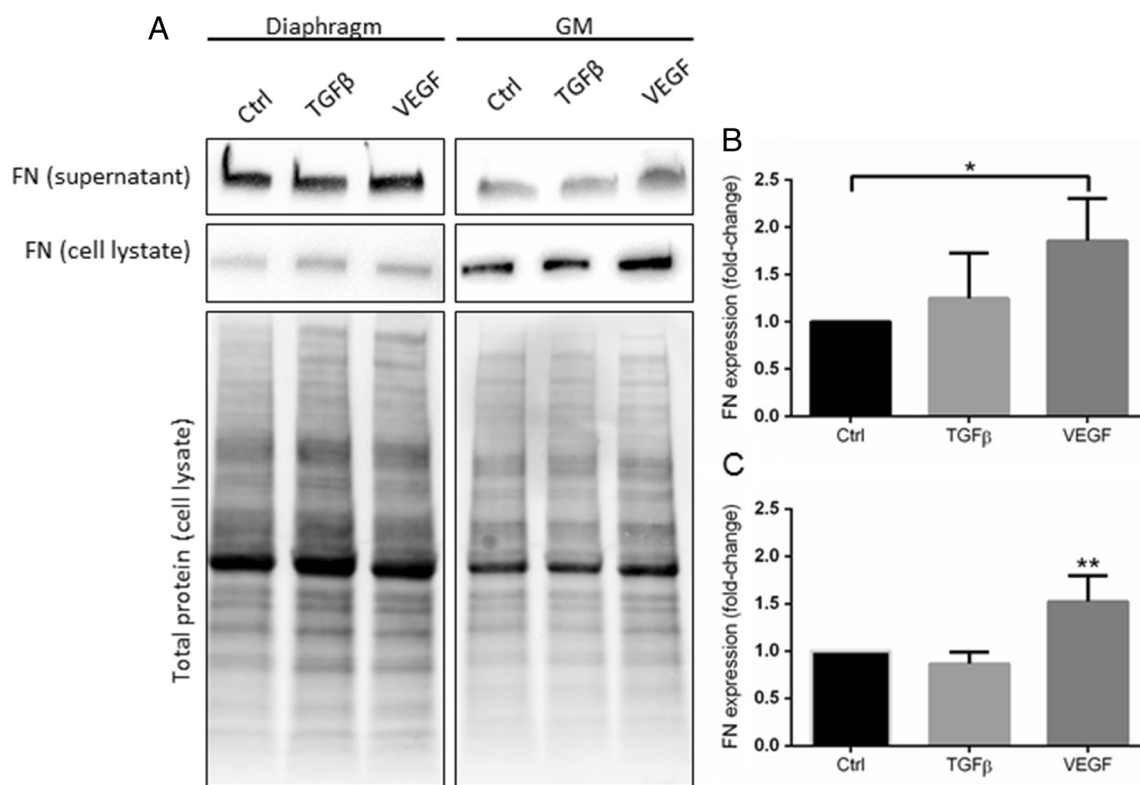


Fig. 2 FN levels are increased following treatment of diaphragm and GM fibroblasts with VEGF. Western blot analysis (a) reveals an increased level of intracellular FN and extracellular FN located in the

cell culture supernatant following 48 h in the presence of VEGF₁₆₄. FN levels from cell lysates of diaphragm (b) and GM (c) fibroblasts were measured relative to total protein ($n=4$, $*p<0.05$, $**p<0.01$)

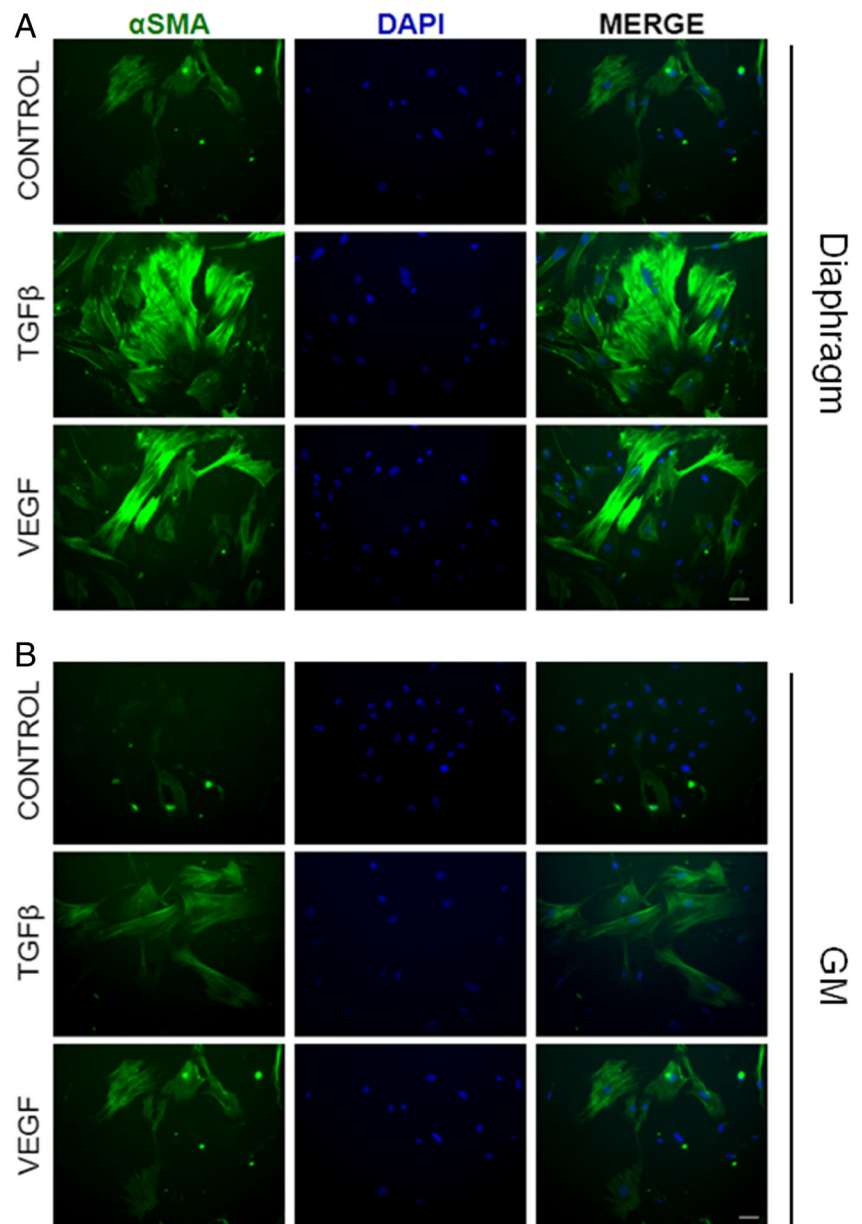
levels of α SMA expression. Myofibroblast differentiation in these cells was evident by stress fiber formation. A similar, but less striking, response was observed in diaphragm fibroblasts treated with VEGF, as a large portion of these cells also underwent stress fiber formation (Fig. 3a). GM-derived fibroblasts appeared to respond to both TGF β and VEGF, although the response was more subdued compared to the one observed in diaphragm fibroblasts (Fig. 3b).

Discussion

Therapeutic approaches to slow or attenuate the degenerative effects of DMD include attempts to restore dystrophin, the cytoskeletal protein that is missing or aberrant in DMD patients (Pessina et al. 2014). Gene and stem cell therapies are but two approaches currently under intense investigation to serve this purpose, but both of these are limited by the hostile microenvironment where regenerative therapy needs to occur. Skeletal muscle in both patients and animal models of DMD is poorly vascularized, leading to studies exploring the use of pro-angiogenic therapies to enhance the ischemic microenvironment (Arsic et al. 2004; Borselli et al. 2010). VEGF is a particularly attractive candidate for such a therapy since it is a well-known, potent inducer of angiogenesis. Although the

literature currently suggests that VEGF enhances cell engraftment in the mdx mouse (Messina et al. 2007; Deasy et al. 2009), it remains to be determined whether VEGF induces a fibrotic response in DMD. In fact, a reduction in fibrosis following delivery of muscle-derived stem cells overexpressing VEGF has been shown in the mdx GM muscle (Deasy et al. 2009). The main motivation for this study was the findings from recent studies that examined the effect of VEGF in other fibrotic diseases such as SSc and idiopathic pulmonary fibrosis, whereby elevated levels of VEGF increased markers of fibrosis. In contrast, inhibition of VEGF was shown to have an anti-fibrotic effect (Maurer et al. 2014; Hostettler et al. 2014). These studies highlight the need to assess angiogenic therapy for the treatment of DMD in an animal model that develops significant fibrosis. Since the mdx mouse does not develop fibrosis as severely and early as DMD patients (Konieczny et al. 2013), our study focused on investigating the effect of VEGF on fibroblasts derived from a more fibrotic mouse model of DMD, the mdx/utrn \pm mouse (Zhou et al. 2008). Furthermore, since most studies to date have focused on the effect of VEGF on the hind limb muscles, our second objective was to determine whether there is a differential response to VEGF in diaphragm and gastrocnemius fibroblasts. To this end, gene expression analyses in the present study have indicated that VEGF treatment may alter expression of some

Fig. 3 VEGF induces stress fiber formation in dystrophic muscle fibroblasts indicative of myofibroblast differentiation. Diaphragm (a) and GM (b) fibroblasts were treated with VEGF₁₆₄ for 48 h. Treatment with TGF β , a known-inducer of pro-fibrotic protein expression, was used as a positive control. Alpha-smooth muscle actin (α SMA), a marker of myofibroblasts, is shown in green. DAPI (blue) was used as a counterstain (scale bar= 50 μ m)



genes involved in the fibrotic response, specifically *Acta2*, the gene that encodes α SMA. Expression of the genes that encode CTGF/CCN2 and collagen were not altered by VEGF, however these findings were inconclusive since we did not further investigate the levels of these two proteins following treatment. Visualization of α SMA expression in VEGF-treated cells suggests a role for this growth factor in promoting differentiation of these fibroblasts, particularly ones derived from the diaphragm, into myofibroblasts.

Although *Fnl1* levels were not affected by either treatment, protein levels of FN increased in the presence of VEGF, but not TGF β . It is perhaps not surprising that exogenous TGF β did not stimulate increased FN levels compared to the control

treatment since all cells in this study were derived from mdx/utrn^{+/-} mice. Previous work has demonstrated that conditioned media from mdx fibroblasts stimulates a fibrotic response, including increased FN, in wild-type fibroblasts (Mezzano et al. 2007). Given these findings, it may be that untreated fibroblasts in the present study also secreted pro-fibrotic factors into the culture medium, leading to increased FN even in the absence of exogenous TGF β . Thus, the finding that exogenous VEGF treatment increased FN levels in our study is particularly intriguing. Since VEGF treatment resulted in increased FN protein but did not alter mRNA expression, it appears that VEGF may act translationally or

post-translationally to enhance FN expression in mdx/utrn^{+/-} fibroblast cultures. The fact that VEGF treatment led to increased FN both in cell lysates and cell culture supernatant warrants further investigation. This finding is consistent with work in other animal models of disease such as preclinical diabetic retinopathy whereby exogenous VEGF injection to a rat retina induced expression of FN as well as other fibrotic markers (Kuiper et al. 2007). That VEGF only affected protein, but not mRNA, levels in the present study also warrants further analysis. Since only two time points were assessed here, looking at FN mRNA expression over a time course may yield different results than those attained in this study. Interestingly, work over the past decade has revealed binding sites for VEGF on full length fibronectin and these studies have suggested that, in this case, the binding of these two proteins in the ECM act to increase the biological activity of VEGF (Wijelath et al. 2002, 2004; Goerges and Nugent 2004; Vempati et al. 2014). Further, it is well known that loss of FN negatively impacts vasculogenesis and angiogenesis due, in part, to the decrease in VEGF activity (Astrof and Hynes 2009). Although previous work highlights an important interaction between FN and VEGF, our results are the first to show a potential effect of VEGF on FN expression. As such, the role of this interaction in the context of angiogenesis in dystrophic muscle also warrants further detailed investigation.

Given that diaphragm and GM muscles in mdx/utrn^{+/-} mice display different abilities to develop fibrosis, we investigated whether fibroblasts from these different muscles also respond differently to VEGF treatment. Although the two cell types showed similar gene expression profiles following treatment, there were a few key differences observed here. First, untreated diaphragm fibroblasts appear to be more myofibroblast-like than GM fibroblasts, as evidenced by the presence of α SMA stress fibers, which were rare in the GM fibroblast cultures. Further, TGF β stimulated a more drastic response in diaphragm fibroblasts compared to GM fibroblasts, evidenced by higher α SMA expressed and more prominent stress fiber formation. A similar trend was observed following VEGF treatment, although not to the same extent as observed following TGF β administration. These findings suggest that fibroblasts from different muscles may respond in a unique way to various growth factors. As such, further work will be needed to determine whether diaphragm-derived fibroblasts indeed respond differently to VEGF treatment than do hind limb muscles. Since the pathology progression is more rapid in the diaphragm than hind limb muscles, considering the effect of treatment on both of these muscles will be crucial for future work.

Acknowledgments We would like to thank Dr. Robert Grange from Virginia Tech, Virginia for generously providing us with the mdx/utrn^{+/-} mice. We would also like to acknowledge Terrie Ann and Dale Forder at the St. Joseph's Animal Care Facility for assistance with animal care and Dr. Justin Crawford for technical assistance.

Author contributions KG carried out the experimental work and performed the statistical analysis. KG and LH conceived and designed the study and drafted the manuscript. Both authors read and approved the final manuscript.

References

- Arsic N, Zacchigna S, Zentilin L, Ramirez-Correa G, Pattarini L, Salvi A et al (2004) Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo. *Mol Ther* 10:844–854
- Astrof S, Hynes RO (2009) Fibronectins in vascular morphogenesis. *Angiogenesis* 12(2):165–175
- Beckman SA, Chen WC, Tang Y, Proto JD, Mlakar L, Wang B et al (2013) Beneficial effect of mechanical stimulation on the regenerative potential of muscle-derived stem cells is lost by inhibiting vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* 33(8):2004–2012
- Bernasconi P, Torchiana E, Confalonieri P, Brugnani R, Barresi R, Mora M et al (1995) Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J Clin Invest* 96(2):1137–1144
- Borselli C, Storie H, Benesch-Lee F, Shvartsman D, Cezar C, Lichtman JW et al (2010) Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. *Proc Natl Acad Sci U S A* 107:3287–3292
- Bouchentouf M, Benabdallah BF, Bigey P, Yau TM, Scherman D, Tremblay JP (2008) Vascular endothelial growth factor reduced hypoxia-induced death of human myoblasts and improved their engraftment in mouse muscles. *Gene Ther* 6:404–414
- Chen YW, Nagaraju K, Bakay M, McIntyre O, Rawat R, Shi R et al (2005) Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. *Neurology* 65(6):826–834
- Deasy BM, Feduska JM, Payne TR, Li Y, Ambrosio F, Huard J (2009) Effect of VEGF on the regenerative capacity of muscle stem cells in dystrophic skeletal muscle. *Mol Ther* 17:1788–1798
- Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L et al (1997) Utrrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell* 90(4):717–727
- Desguerre I, Mayer M, Leturcq F, Barbet JP, Gherardi RK, Christov C (2009) Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation. *J Neuropathol Exp Neurol* 68:762–773
- Desguerre I, Arnold L, Vignaud A, Cuvellier S, Yacoub-Youssef H, Gherardi RK et al (2012) A new model of experimental fibrosis in hindlimb skeletal muscle of adult mdx mouse mimicking muscular dystrophy. *Muscle Nerve* 45(6):803–814
- Goerges AL, Nugent MA (2004) pH regulates vascular endothelial growth factor binding to fibronectin: a mechanism for control of extracellular matrix storage and release. *J Biol Chem* 279:2307–2315
- Goyenvallé A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD et al (2010) Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. *Mol Ther* 18(1):198–205
- Goyenvallé A, Babbs A, Wright J, Wilkins V, Powell D, Garcia L et al (2012) Rescue of severely affected dystrophin/utrophin-deficient

- mice through scAAV-U7snRNA-mediated exon skipping. *Hum Mol Genet* 21(11):2559–2571
- Grady RM, Merlie JP, Sanes JR (1997a) Subtle neuromuscular defects in utrophin-deficient mice. *J Cell Biol* 136(4):871–882
- Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR (1997b) Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell* 90(4):729–738
- Gregorevic P, Blankinship MJ, Allen JM, Crawford RW, Meuse L, Miller DG et al (2004) Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* 10(8):828–834
- Gutpell KM, Hrinivich WT, Hoffman LM (2015) Skeletal muscle fibrosis in the mdx/utrn^{+/-} mouse validates its suitability as a murine model of Duchenne muscular dystrophy. *PLoS ONE* 10(1):e0117306
- Hostettler KE, Zhong J, Papakonstantinou E, Karakiulakis G, Tamm M, Seidel P et al (2014) Anti-fibrotic effects of nintedanib in lung fibroblasts derived from patients with idiopathic pulmonary fibrosis. *Respir Res* 15:157–166
- Huang P, Cheng G, Lu H, Aronica M, Ransohoff RM, Zhou L (2011) Impaired respiratory function in mdx and mdx/utrn^{+/-} mice. *Muscle Nerve* 43(2):263–267
- Konieczny P, Swiderski K, Chamberlain JS (2013) Gene and cell-mediated therapies for muscular dystrophy. *Muscle Nerve* 47(5):649–663
- Kuiper EJ, Hughes JM, Van Geest RJ, Vogels I, Goldschmeding R, Van Noorden C et al (2007) Effect of VEGF-A on expression of profibrotic growth factor and extracellular matrix genes in the retina. *Invest Ophthalmol Vis Sci* 48(9):4267–4276
- Maurer B, Distler A, Suliman YA, Gay RE, Michel BA, Gay S et al (2014) Vascular endothelial growth factor aggravates fibrosis and vasculopathy in experimental models of systemic sclerosis. *Ann Rheum Dis* 73(10):1880–1887
- Messina S, Mazzeo A, Bitto A, Aguenouz M, Migliorato A, De Pasquale MG et al (2007) VEGF overexpression via adeno-associated virus gene transfer promotes skeletal muscle regeneration and enhances muscle function in mdx mice. *FASEB J* 21(13):3737–3746
- Mezzano V, Cabrera D, Vial C, Brandan E (2007) Constitutively activated dystrophic muscle fibroblasts show a paradoxical response to TGF- β and CTGF/CCN2. *J Cell Commun Signal* 1(3):205–217
- Miyazaki D, Nakamura A, Fukushima K, Yoshida K, Takeda S, Ikeda S (2011) Matrix metalloproteinase-2 ablation in dystrophin-deficient mdx muscles reduces angiogenesis resulting in impaired growth of regenerated muscle fibers. *Hum Mol Genet* 20(9):1787–1799
- Pessina P, Cabrera D, Morales MG, Riquelme CA, Gutiérrez J, Serrano AL et al (2014) Novel and optimized strategies for inducing fibrosis in vivo: focus on Duchenne muscular dystrophy. *Skelet Muscle* 4:7
- Rhoads RP, Flann KL, Cardinal TR, Rathbone CR, Liu X, Allen RE (2013) Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. *Biochem Biophys Res Commun* 440(3):399–404
- Shimizu-Motohashi Y, Asakura A (2014) Angiogenesis as a novel therapeutic strategy for Duchenne muscular dystrophy through decreased ischemia and increased satellite cells. *Front Physiol* 5:50
- Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B et al (1991) The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 352(6335):536–539
- Taylor SC, Berkelman T, Yadav G, Hammond M (2013) A defined methodology for reliable quantification of western blot data. *Mol Biotechnol* 55(3):217–226
- van Putten M, Kumar D, Hulsker M, Hoogaars WM, Plomp JJ, van Opstal A et al (2012) Comparison of skeletal muscle pathology and motor function of dystrophin and utrophin deficient mouse strains. *Neuromuscul Disord* 22(5):406–417
- Vempati P, Popel AS, Mac Gabhann F (2014) Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning. *Cytokine Growth Factor Rev* 25(1):1–19
- Wijelath ES, Murray J, Rahman S, Patel Y, Ishida A, Strand K et al (2002) Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res* 91:25–31
- Wijelath ES, Rahman S, Murray J, Patel Y, Savidge G, Sobel M (2004) Fibronectin promotes VEGF-induced CD34 cell differentiation into endothelial cells. *J Vasc Surg* 39:655–660
- Zhou L, Rafael-Fortney JA, Huang P, Zhao XS, Cheng G, Zhou X et al (2008) Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in mdx mice. *J Neuro Sci* 264(1–2):106–111