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VEGFA promotes skeletal muscle regeneration in aging

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Abstract

Aging is associated with a loss of skeletal muscle regeneration. We hypothesized that differentially regulated vascular endothelial growth factor (VEGF)A with aging partially underlies this loss of regenerative capacity. To assess the role of VEGFA in muscle regeneration, young (12–14 weeks old) and old C57BL/6 mice (24–25 months old) were subjected to cryoinjury in tibialis anterior (TA) muscle to induce muscle regeneration. The average cross-sectional area (CSA) of regenerating myofibers was 33% smaller in old as compared to young (p<0.01) mice, which correlated with a 2-fold loss of muscle VEGFA protein levels (p=0.02). The capillary density in the TA was similar between the two groups. To assess effects of VEGF on muscle cells, siRNA was utilized to knockdown VEGF expression in C2C12 myoblasts and resulted in impaired differentiation in comparison to control groups. Young VEGF^{lo} mice, with a 50% decrease in systemic VEGFA activity, exhibited a 2-fold reduction in the average regenerating fiber CSA following cryoinjury (p<0.01) in comparison to their littermate controls. ML228, a hypoxia signaling activator known to increase VEGFA levels, augmented muscle VEGFA levels 2-fold and increased average CSA of regenerating fibers in both old mice (25% increase, p<0.01) and VEGF^{lo} (20% increase, p<0.01) mice, but not in young or the littermate WT controls. A marked fatty deposition in the regenerating area noted in the VEGF^{lo} group, which was significantly reduced with ML228 supplementation. These results suggest that VEGFA may be a therapeutic target in age-related muscle loss.

Graphical Abstract.

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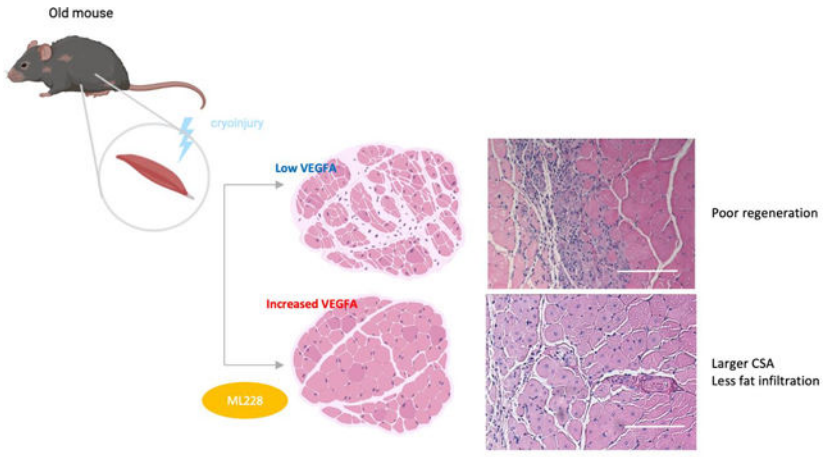

Aging is associated with a loss of skeletal muscle regeneration with a concurrent reduction in VEGFA. Low VEGF levels impairs myoblast differentiation in vitro, and does not seem to affect muscle stem cell (MuSC) activation / proliferation in vivo. Pharmacological restoration of VEGFA level with ML228 in skeletal muscle of old mice results in a significant improvement in muscle regeneration.

Keywords

muscle regeneration; aging; sarcopenia; hypoxia signaling

Introduction

Skeletal muscle possesses a remarkable ability to regenerate and repair itself following injuries. However, the regenerative potential of muscle declines with aging.[1,2] Skeletal muscle stem cells (muSCs), which reside beneath the basement membrane on mature myofibers, are primarily responsible for skeletal muscle regeneration and efficient muscle repair following injury in adult.[3,4] With normal aging, the myogenic potential of muSCs decreases and may potentially lead to aging-associated loss of skeletal muscle mass.[5] The mechanisms underlying this age-related decline in muscle regenerative function remains largely undefined to this date; however, identifying the key pathways and effector molecules differentially regulated in aging may offer attractive targets for improving muscle regeneration and potentially for the treatment of sarcopenia, or aging-related muscle loss.[6–8]

With aging, both intrinsic changes in muSCs and extrinsic changes within the stem cell niche contribute to impaired muscle regeneration.[9–11] Within skeletal muscle, satellite cells exist in the niche, or microenvironment near the basal lamina, and are influenced by a local milieu of growth factors released by interstitial cells, blood vessels, motor neurons, myofibers, as well as adjacent satellite cells.[11] As such, differentially regulated factors in skeletal muscle with aging are potential targets to restore skeletal muscle regenerative potential. Vascular endothelial growth factor (VEGF)A, in particular, is known to promote skeletal muscle regeneration and VEGF signaling insufficiency occurs with aging across multiple organs, including skeletal muscle.[12] Hypoxia signaling, which

decreases substantially in skeletal muscle with aging, may underlie this loss of VEGFA.[13] However, the role of VEGF signaling with regards to aging and muscle regeneration has not been previously characterized.

Multiple studies suggest VEGF is necessary for skeletal muscle homeostasis and muSC function. Skeletal muscle tissue is the most abundant producer of VEGFA in the body and its levels are higher in skeletal muscle than other VEGF isoforms. It has already been extensively studied in the skeletal muscle fibers in models of VEGFA knockout mice,[14–18] as well as VEGFA overexpression.[19–21] Collectively, these studies demonstrate the importance of VEGFA in skeletal muscle maintenance. Another study has demonstrated that the loss of VEGFA in muSCs limits their engraftment potential in dystrophic mice, and deletion or blockade of VEGF interaction with its decoy receptor Flt resulted in functional improvement in mdx dystrophic mice.[22,23] Other studies have suggested that topical application of VEGF improves strength recovery and limits fibrosis following muscle injury.[24] Separately, VEGF supplementation improved myofiber hypertrophy *in vitro* and increases the number of regenerating fibers in both normally perfused and ischemic muscle.[25] Conversely, muscle hypertrophy in response to loading is limited with VEGF inhibition.[26] Furthermore, VEGF deletion or pharmacological inhibition in muSCs have been shown to reduce their proximity to capillaries in vivo, which is essential for the maintenance of MuSC quiescence, which in turn dictates the regenerative capacity of muSCs.[27] Taken together, these studies suggest that loss of VEGF may underlie some of the loss of myogenic potential in muSCs that occurs with aging. Given this, we hypothesized VEGF, a potent downstream signal well characterized in nascent vascular ingrowth and progenitor differentiation, is differentially regulated in aging [28,29] and may contribute to differences in regeneration seen in young and old muscle.

We found that skeletal muscle regeneration is significantly decreased in old mice, in corollary with decreased skeletal muscle VEGFA levels. In a myoblast cell line, knockdown of VEGFA diminished myofiber tube size *in vitro*. Next, using a genetically modified mouse model with systemically decreased levels of VEGFA,[30] we found skeletal muscle regeneration was similarly decreased. Use of ML228, a hypoxia pathway activator that promotes VEGFA levels,[21] reverses this loss of skeletal muscle myogenic potential in both old mice and mice with decreased VEGFA activity. Taken together, these experiments suggest that differential regulation of VEGFA, as occurs with aging, may impact loss of myogenic potential in skeletal muscle.

Methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Brigham and Women's Hospital and were conducted within the guidelines of the US National Institutes of Health. Young C57BL/6 mice (12–14 weeks of age) were obtained from Jackson Laboratories. Old C57BL/6 (24–25 months of age) mice were obtained from the National Institute on Aging. $VEGF^{lo}$ mice (STOCK Vegfatm2.1Nagy/J) (12–14 weeks old) were procured from Jackson Laboratory (Stock No: 027315). In brief, these 129S1/SvImJ mice carry knock-in mutations of the *Vegfa* gene, which yields a gene product with

reduced VEGFA activity (25–50% reduction).[26] In experiments involving ML228 treatment, ML228 (TOCRIS #1357171-62-0, Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMSO (0.03 mg ML228 in 1 mL DMSO) and was injected intraperitoneally (0.1 mL) once daily for a total of 5 days.[21] Cryoinjury was induced on the left legs of the animals immediately following the second injection of DMSO, as a vehicle control, or ML228 approximately 24 hours following the first injection.[31] The animals were housed at the Brigham and Women's Hospital Animal Care Facility and were given ad libitum access to food and water. An equal number of male and female mice were utilized for all experiments.

2.2. Muscle Cryoinjury and Assessment of Skeletal Muscle Regeneration

All mice were anesthetized using isoflurane. A skin incision was made on the lower leg and the tibialis anterior (TA) was exposed. A metal probe cooled in dry ice was placed on the skin for 10 seconds, creating a cryoinjury.[31] Injured muscles were then allowed to recover for 5 or 10 days before harvest. Cryoinjury generates a reproducible, uniform injury in the muscle with a discrete border between uninjured and injured muscle, which remains distinct when evaluating the regenerative area. To quantify the area of regeneration, 10 photos per sample were taken spanning the entire regenerating area in cross section, and cross-sectional area (CSA) of 100 regenerating myofibers (identified by their centrally located nuclei) were measured using ImageJ. The CSA was then averaged to establish a mean CSA of regenerating fibers for each mouse. The CSA means were then used to compared between groups, as previously described.[31,32]

2.3. Histological Analysis

Muscles were fixed in 4% of formalin, washed in PBS and stored in 70% of ethanol for paraffin embedding. Hematoxylin and eosin (H&E) staining was used to evaluate the regenerating area. Newly formed myofibers were identified by their centrally located nuclei. All CSA measurements were completed in a blinded fashion and performed by two separate technicians. For immunostaining, skeletal muscle sections were blocked with Blocker BSA (#37520, ThermoFisher, Waltham, MA, USA), and stained with antibodies against CD31 (1:100, #ab7388, Abcam) or Pax7 (1:100, # PA5-68506, Thermo Fisher Scientific, USA) and Laminin (1:100, #ab11571, Abcam, Cambridge, UK). Fat infiltration was estimated as previously described using a commonly employed "pixel and area" classification steps on ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).[33] Briefly, the H&E cross-sectional images of injured muscles were converted into pixels and the background pixels were identified by their color. The area of the background pixels that presented fat droplets were identified by their eccentricity. The adjacency statistics were applied to the results to increase the accuracy of fat identification. This was performed on 10 non-overlapping, randomly selected images spanning the regenerating area per samples, and the average infiltration was estimated as a percentage of the total area of the tissue. Separately, C2C12 cells were stained with antibodies against myosin heavy chain (MF20) (1:100, #14-6503-82, ThermoFisher) and DAPI. Secondary antibodies used were (1:400, #A21470, ThermoFisher) and (1:400, #A11037, ThermoFisher), respectively. Bright field and fluorescent images were acquired using an Olympus BX53 microscope. 20X images were taken throughout the sectioned muscle. Within these high-powered fields, CD31 positive cells were quantified and normalized to the number of fibers within the field.

Pax7-positive signals were counted per high-power-field, and the average numbers of signal per 10 images per sample were calculated. Fusion index of C2C12 cells after differentiation were calculated using the following formula:

$$Fusion\ Index\ (\%) = \frac{Total\ nuclei\ -\ nuclei\ outside\ of\ MF20\ postive\ cells\ -\ nuclei\ in\ MF20\ positive\ cells\ with\ 1\ or\ 2\ nuclei\ \#}{Total\ number\ of\ nuclei} \times 100$$

2.4. Protein quantification

Whole muscle lysates were isolated from TA muscles using lysate buffer (Phosphosolutions, #SKU-100Lys) and subjected to immunoblotting and Elisa. For immunoblotting, proteins were separated on a 4%-12% of SDS-polyacrylamide gel with MOPS SDS Running Buffer (ThermoFisher) at 150 V. The gel was transferred onto an Immuno-Blot PVDF Membrane using the iBlot2 Dry Blotting System (ThermoFisher) for 7 minutes. The membranes were then blocked in blocking solution (Life Technologies, Carlsbad, CA) for 1 hour at room temperature then incubated with an antibody overnight at 4°C with constant shaking. The antibody utilized was VEGFA (Abcam ab46154). Following three 5-minutes washes in TBS-T buffer, the membranes were incubated with anti-rabbit IgG HRP-linked (Cell Signaling) secondary antibody. All the antibodies were diluted in blocking buffer. For immunodetection, the membranes were washed three times with TBS-T buffer, incubated with ECL solutions per manufacturer's specifications (Amersham Biosciences, Little Chalfont, UK) and exposed to Hyperfilm ECL. The membranes were stripped and re-probed with an antibody recognizing GAPDH for normalization. Band intensities were determined using ImageJ software. Elisa was performed as per manufacturer's instructions (Abcam ab209882), and the microplates were read at 450nm for OD. The concentration of VEGF protein in the samples were determined by interpolating the blank control subtracted absorbance values against the standard curve.

2.5. C2C12 siRNA treatment

C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C with 5% CO2 supply in a humidified incubator. At 75% confluency, cells were induced to undergo differentiation by adding DMEM supplemented with 2% horse serum for 5 days. Media was replaced every 24 hours during differentiation. For siRNA transfections, C2C12 cells were seeded in 6-well plates without coverslips and differentiated when 75% confluent. Cells were transfected every 24 hours from the day of adding the differentiation media referred to as Day 0 till Day 5 of differentiation. For each well, 150 uL of OPTI-MEM media (Life Technologies) was mixed with 3 μL of 10 uM siRNA (siControl or siVEGF from Santa Cruz Biotechnology, Dallas, TX, ISA) and 9 μL of transfection reagent Lipofectamine RNAiMAX (Life Technologies) in separate tubes and incubated for 5 minutes. After 5 minutes, 150 μL of the constituent solutions from each tube were combined and incubated for another 30 minutes at room temperature to generate 300 μL of transfection mix. A 250 μL of the transfection mix were added per well to have a final siRNA concentration of 11 nM per well. Cells were collected from Day 0 (undifferentiated and non-transfected control)

to Day 5. Cells were harvested in 1 mL TRIzol for RNA extraction or fixed on coverslips with 4% PFA for immunofluorescence staining.

2.6. Quantitative RT-PCR

mRNA was extracted from C2C12 cells was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from mRNA using SuperScript III First-Strand kit (Invitrogen). Real-time quantitative PCR reactions were carried out in ABI StepOnePlus machine, using SYBR Green PCR mix (ThermoFisher). The temperature profile used was 95 degrees for 0 minutes, then, 40 cycles of 95 degrees C, 60 degrees C, 72 degrees C each for 30 seconds. All the gene expression levels were normalized to β-actin. Primers sequences are: VEGF-A and β-actin: F: (5′ CCTAAGGCCAACCGTGAAAA 3′) and R: (5′ AGCCATACAGGGACAGCACA 3′).

2.7. Statistical Analysis

Data are presented as the mean ± standard error. Statistical comparisons for normally distributed data were assessed for statistical significance using Student's t-test. Results comparing more than two groups were assessed by one-way ANOVA with Tukey's multiple comparison test (GraphPad Prism, GraphPad Software Inc, San Diego, CA, USA, RRID: SCR_002798). The investigators were blinded to the experimental group assignments for outcome assessment. Statistical significance was accepted at $p<0.05$.

Results:

Skeletal muscle regeneration and whole muscle VEGFA are reduced with aging.

To assess skeletal muscle regeneration, both young and old mice were subjected to cryoinjury of the TA. Old mice demonstrated markedly impaired regenerative response, as indicated by a significantly smaller myofiber CSA in comparison to that of young at both 5 (507±14 vs 386±10μm^2 in young and old mice, respectively, $p<0.001$) and 10 days post injury (DPI) (1251±66 vs 833±18μm^2 in young and old mice, respectively, $p<0.001$) (Figure 1A–C). The density of Pax7-positive satellite cells in the regenerating area was found to be significantly lower in old mice on DPI 10 compared to young mice (Figure D,E). Next, we evaluated the whole muscle VEGFA protein levels given its implicated role in skeletal muscle regeneration and our previous data demonstrating hypoxia signaling in skeletal muscle decreases with aging. VEGFA in the TA was found to be 1.5-fold lower in old as compared to young mice by immunoblotting at baseline (p=0.02) and 2-fold lower on DPI 5 (p=0.02) and 10 (p=0.02) (Figure 1F–I, Supplemental Figure 1A,B). The whole muscle VEGF levels were similar between the baseline and DPI10 timepoints in both young and old mice. (Supplemental Figure 1C). The differences in the VEGFA levels within skeletal muscle further prompted us to determine whether there were any effects on muscle angiogenesis, which depends on VEGF signaling. At the ages tested, the capillary-to-muscle-fiber ratio were similar between the two groups both at the baseline, (p=0.9) (Figure 1J,K), and on DPI 10 (p=0.42) (Supplementary Figure 2A,B).

VEGFA inhibition leads to poor skeletal muscle differentiation *in vitro*.

Next, to establish whether the loss of VEGFA is sufficient to limit differentiation in a myoblast cell line, C2C12 cells were subjected to small interfering (si)RNA to impair normal secretion of VEGFA. siRNA treatment of C2C12 cells resulted in 98±5% reduction of VEGFA expression in C2C12 cells, as compared to treatment with scrambled siRNA (n=4 per group, $p<0.001$). Treatment with VEGFA siRNA resulted in a 54% reduction ($p<0.001$) in muscle fiber diameter (Figure 2A,B), and 45% reduction (p=0.003) in fusion index (Figure 2C) in comparison to the control group treated with scrambled siRNA on day 5 differentiation. The total nuclei numbers were similar in both group on day 5 differentiation (p=0.3) (Figure 2D).

Systemic reduction of VEGFA impairs skeletal muscle regeneration.

To determine whether loss of VEGFA activity within skeletal muscle, as occurs with aging, is sufficient to impair muscle regeneration, we utilized genetically modified mice with a marked loss of VEGFA activity. Heterozygote mice carry a mutation within exon 8 which is partially replaced by the *lacZ* gene leading to downstream aberrant splicing events. These mutant transcripts are detectable by RT-PCR and precipitate a reduction in protein activity of 25–50%.[30] All $VEGF^{lo}$ mice and littermate controls were 20 weeks old at the time of experimentation. In comparison to littermate controls, the levels of the normal VEGFA in skeletal muscle of $VEGF^{lo}$ mice are decreased by 61% compared to that of the littermate controls ($p=0.01$) at baseline and 56% following injury ($p<0.001$) (Figure 3A–D). Baseline CSA of uninjured muscle fibers were similar between the two groups (Supplemental Figure 4). Of note, the absolute regenerating fiber size is smaller overall in these 129S1/SvImJ mice as compared to the aging experiments, which utilized C57BL/6 mice.

Following cryoinjury, $VEGF^{lo}$ mice exhibited poor regeneration, with a 2-fold decrease in CSA of regenerating fibers on DPI 10 ($p= 0.001$) (Figure 3G,H,J). In addition to the impairment in skeletal muscle regeneration, substantial fat deposition within the regenerating area was noted in the $VEGF^{lo}$ mice as compared to littermate controls on DPI10 (Estimated 11±2 vs 53±5% of fat within the regenerating area, control and $VEGF^{lo}$ mice, respectively, $p<0.001$) (Figure 3I). Capillary-to-muscle-fiber ratio was evaluated by CD31 staining, and found to be similar in muscles at the baseline in both VEGF WT and $VEGF^{lo}$ mice (2.2±0.1 vs 2.0±0.1 capillaries/fiber, control and $VEGF^{lo}$ mice, respectively, $p=0.14$) (Figure 3E,F) and on DPI10 (0.6±0.02 vs 0.65±0.02 capillaries/fiber, control and $VEGF^{lo}$ mice, respectively. p=0.42) (Supplemental Figure 3A,B).

Pharmacological stimulation of VEGFA improves skeletal muscle regeneration in old and $VEGF^{lo}$ mice.

To determine whether restoration of VEGF can improve skeletal muscle regeneration in old mice, ML228, a HIF pathway activator known to increase VEGFA levels,[29] or vehicle control (DMSO), was administered prior to and immediately following cryoinjury once daily, for 5 days total. ML228 is a small molecule activator of the hypoxia inducible factor-pathway, which induces HIF1-α nuclear translocation and stimulates VEGF production. Previous studies suggest that ML228 augments VEGF-A protein levels in both rodent[25] and murine models.[13] ML228 supplementation improved skeletal muscle regeneration in old

mice by 44% as compared to age-matched controls treated with vehicle ($p<0.001$) (Figure 4A,B). Following ML228 treatment, VEGFA protein levels increased by 3 folds at baseline compared to the DMSO treated littermate controls, ($p<0.001$) and remained elevated by 2 folds on DPI10 ($p<0.001$) (Figure 4C–F). Next, we sought to determine whether augmentation of functional VEGFA improves skeletal muscle regeneration in $VEGF^{lo}$ mice. ML228 supplementation augmented the average regenerating fiber CSA in $VEGF^{lo}$ in comparison to the $VEGF^{lo}$ mice treated with DMSO vehicle (661±84 μm^2 vs 341±10μM, respectively, $p=0.02$) (Figure 5A,B). This was accompanied by a significant reduction in intramuscular fat (37±2 vs 24±3% of fat within the regenerating area in VEGFlo mice treated with ML228 vs DMSO, respectively, $p=0.02$) (Figure 5C). Treatment of VEGF WT mice with ML228 did not improve regeneration in comparison to treatment with vehicle (Figure 5A,B). Similarly, ML228 supplementation elevated the functional VEGFA levels in $VEGF^{lo}$ mice by nearly 2 folds ($p=0.04$), but not in VEGF WT mice, in comparison to DMSO treated control groups (Figure 5D,E). Capillary densities were found to be similar across all the groups tested (Supplementary Figure 5).

Discussion

Loss of skeletal muscle regeneration with aging may contribute to loss of muscle mass and function.[35] Other studies have demonstrated that preservation of muscle SC function to replenish muscle fibers throughout aging may be sufficient to maintain muscle mass and limit the development of sarcopenia.[36] Although the loss of muscle's ability to regenerate with aging is likely multifactorial, changes in the intrinsic signaling in stem cells and the stem cell niche such as loss of growth factors, altered blood supply, and increased local inflammation are major determinants of skeletal muscle regenerative capacity.[36]

Previous studies demonstrated that hypoxia signaling is differentially regulated with aging[21] and VEGFA, a key target of the hypoxia signaling pathway,[37] may regulate skeletal muscle regeneration.[25] We therefore hypothesized that the loss of VEGFA within skeletal muscle with aging may be responsible for the loss of regenerative potential with aging. Here, we report that VEGFA levels are significantly less in the skeletal muscle of old mice and that loss of VEGFA is correlated with poor skeletal muscle regeneration in both old mice and mice with systemically reduced VEGFA activity. The restoration of VEGFA, with the use of a pharmacological hypoxia pathway activator, ML228, improved skeletal muscle regeneration in both old and $VEGF^{lo}$ mice, suggesting that VEGFA may indeed have a role is skeletal muscle regeneration and its decline seen in aging. ML228 increased the whole muscle VEGFA levels, but it should be noted that ML228 also modulates hypoxia signaling in general. As such, we cannot definitely conclude that ML228 improves skeletal muscle regeneration in old and VEGFlo mice purely through a VEGFA-mediated pathway.

In order to determine the extravascular role of VEGFA on myofiber differentiation, we utilized a myoblast line and treated with siRNA to interfere with VEGFA expression during induced differentiation in differentiation media. VEGFA siRNA treatment resulted in a 2-fold reduction in fiber diameter and fusion index following differentiation *in vitro* without significantly affecting proliferation. These results suggest that production of VEGFA is required for normal differentiation of myoblasts into myofibers, and that

differentiating myoblasts may act as a source of VEGFA that serves as a paracrine regulator of nearby satellite cells. Overall, this support previous studies demonstrating the ability of VEGFA to exert extra-angiogenic effects on myogenesis, including promotion and hypertrophy and fusion following injury.[25] Similarly, others have observed that VEGFA supplementation promotes muscle function and myoglobin expression in ischemic skeletal muscle, independent of angiogenesis.[38] In contrast to the reduction in the Pax7-positive satellite cell density observed in the regenerating area in old mice, $VEGF^{lo}$ mice exhibited a comparable satellite cell density in the regenerating area to that of the control mice. These results, together with the reduced regenerating myofiber CSA observed in $VEGF^{lo}$ mice, further suggest that the loss of VEGF affects muscle regeneration by hindering the differentiation and fusion of myofibers, rather than MuSC activation and proliferation.

It remains difficult, however, to determine the role of capillary density and vascular flow independently from the contribution of direct VEGFA effects. In a trauma model involving tibial fracture, muscle functional recovery was improved by VEGF supplementation,[24] but was accompanied with significant new blood vessel formation in the limb. Within ischemic limbs, VEGFA supplementation promoted functional muscle recovery in conjunction with angiogenesis when delivered in a sustained release fashion.[39] Interestingly, VEGF production is dramatically increased within mature myofibers and regenerating myotubes, suggesting VEGF, via the VEGFR2 receptor, may be involved in muscle recovery and function.[40] Our study similarly suggests that VEGF promotes skeletal muscle regeneration, although the primary source of VEGF within skeletal muscle remains unclear. In addition, further studies are necessary to characterize the relative contributions of VEGF treatment on myogenesis and angiogenesis, and evaluate the effects of angiogenesis and blood flow on muscle regeneration. It should be noted, however, that capillary density within skeletal muscle was not different between the young and old mice at the ages tested, nor $VEGF^{lo}$ mice and littermate controls in uninjured and injured muscles. This suggests VEGF may influence myogenesis, independent from its angiogenic effects.”

Interestingly, loss of VEGF resulted in dramatic fat deposition within the regenerating areas of muscle. Multiple studies have shown that Intermuscular adipose tissue (IMAT) increases with aging at both baseline and following injuries, although it was not as pronounced in the old mice in the present study.[41,42] Interestingly, others have reported that upregulation of the hypoxia signaling pathway increases hepatic steatosis, mediated by both HIF-1α[43]and HIF-2α[44]. In skeletal muscle, HIF-1α improves glucose metabolism and insulin sensitivity,[45] thereby potentially decreasing the risk of IMAT, but the role of hypoxia signaling and VEGF in IMAT remains unclear. While the source of adipocytes observed in $VEGF^{lo}$ mice is unknown, in the context of muscle regeneration, most adipogenesis occurs as a result of resident fibro-adipogenic progenitors (FAPs) expansion and differentiation. FAP expansion is an integral step in muscle repair, and plays an essential role in the regulation of myogenesis by influencing the myogenic behavior and the fate of SCs.[46–48] FAP expansion occurs immediately following muscle injury, releasing cytokines and growth factors which in turn promote cell proliferation / survival but may block differentiation of myogenic cells. The subsequent reabsorption of FAPs is required to allow SC differentiation, fusion and formation of mature myofibers, and consequently, successful myogenesis.[49] Myofibers in turn suppress the proliferation of FAPs, thus preventing further FAP mediated

adipogenesis and fibrosis.[50,51] Marked fat deposition observed in muscles containing lower levels of VEGFA may be a result of any one or the combination of abnormal FAP expansion, increased adipogenic differentiation or failure of FAP clearance. Loss of the inhibitory regulation on FAP-mediated adipogenesis due to failure in myofiber differentiation / maturation may offer a plausible mechanical explanation for the association between lower muscle VEGFA levels and increased fat deposition. Evidently, supplementation with ML228, a hypoxia signaling activator, resulted in reduced intramuscular fat deposition in the $VEGF^{lo}$ mice, with concurrent improvement in muscle regeneration. Whether this is a direct effect of lower levels of VEGF, or indirect effect through hypoxia signaling activation due to loss of negative feedback or poor vascularization of muscle remains to be determined, but that is beyond the scope of this study.

In addition to the limitations mentioned in the above section, further studies will be required to determine if poor myogenesis observed in the $VEGF^{lo}$ mice is due to the niche effects of lower whole muscle VEGF on SC or alterations in the intrinsic functions of SC themselves. In addition, the mechanism by which VEGFA improves skeletal muscle regeneration remains unclear. Previous studies suggest that VEGF can improve myoblast proliferation and survival following injury, suggesting that VEGF may promote a relative abundance of local muSC immediately following injury, thereby promoting improved regeneration.[52] Separately, it may be possible that VEGF modulates key myogenic factor levels within skeletal muscle, such as Notch,[53] which is required for muscle regeneration. Further research is necessary to elucidate mechanistically how restoration of VEGFA in muscle promotes regenerative capacity. Furthermore, VEGFA is abundantly produced within skeletal muscle, but we cannot rule out the contribution of other cell types of muscle VEGFA levels and how their expression of VEGFA may be differentially regulated in aging. In addition, a further study is required to evaluate any changes in the VEGF receptor expressions and their downstream effects to better characterize the relationships between age-related changes in VEGF signaling and regenerative response of skeletal muscles.

However, taken together, our data suggest that VEGF levels decline with aging and this may be partially responsible for a corollary loss of skeletal muscle regeneration. Pharmacological restoration of VEGF improves muscle regeneration, both in old mice and mice with a systemic reduction in VEGF levels and activity. These findings suggest that restoration of VEGF signaling within skeletal muscle may assist the maintenance of skeletal muscle mass and myogenic potential in aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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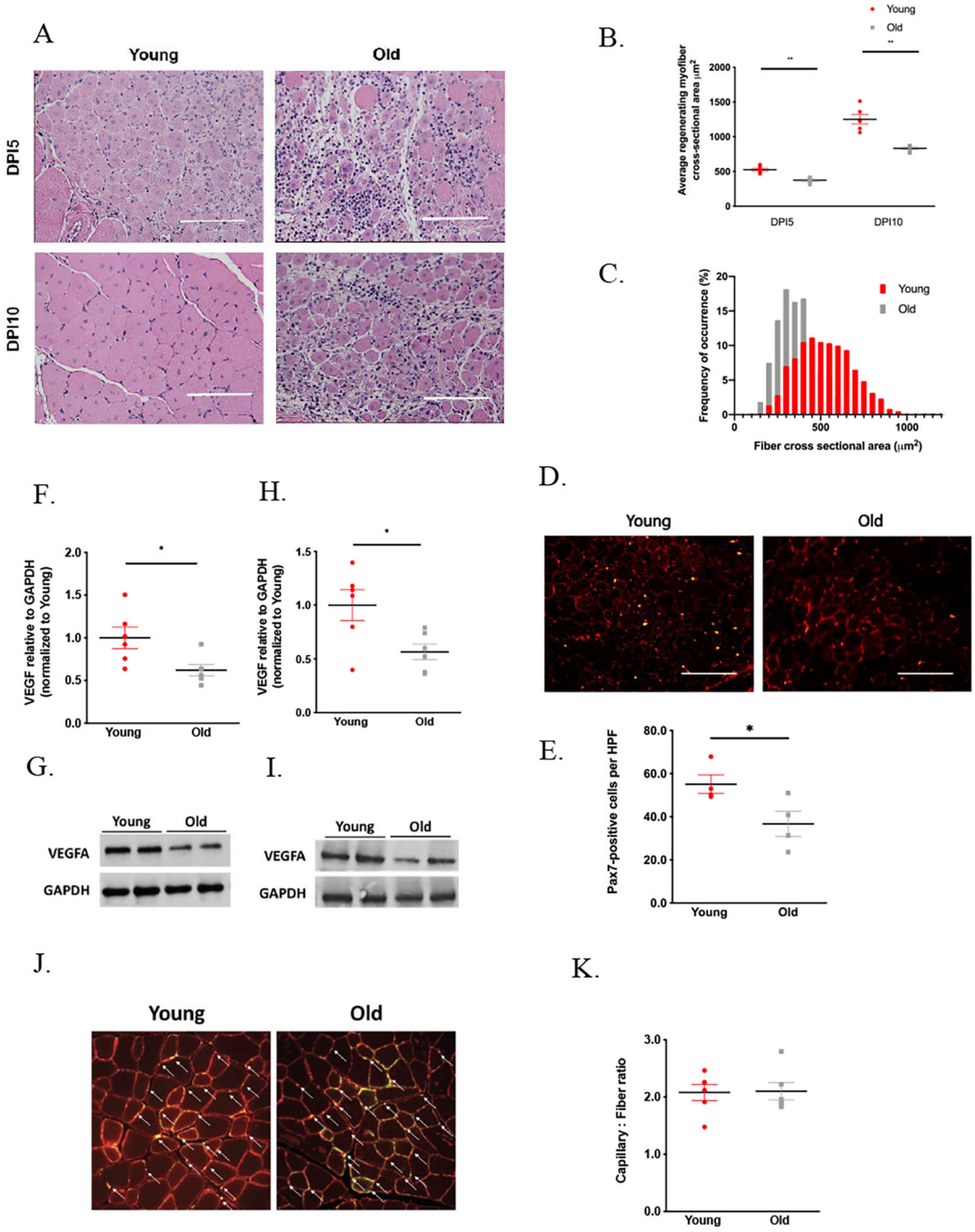

Figure 1. Skeletal muscle regeneration decreases with aging and in correlation with loss of whole muscle VEGF

A. Young (12–14 weeks old) and Old (24–26 months old) were subjected to tibialis anterior cryoinjury and evaluated for size of regenerating fibers by histology. H&E staining of cross sections of muscles from young and old mice 5 and 10 days following cryoinjury. Scale bar = 100 μm. (**A**). Young mice exhibited significantly larger regenerating fiber size as well as fiber organization on both DPI 5 (525.5±21.45 vs 373.4±15.51 μm^2 for young vs old, n=6 each, $p<0.001$) and 10 (1251.0±66.75 vs 833.3±44.82 μm^2 for young vs old, n=6 each,

p<0.001) (**A-C**). Whole muscle VEGFA levels are decreased by approximately 2-fold in skeletal muscle of old mice, both at baseline (1.00±0.13 vs 0.62±0.07 for young vs old, n=6 each, p=0.02) (**F,G**) and following skeletal muscle cryoinjury (1.00±0.14 vs 0.56±0.07 for young vs old, n=6 each, p=0.02) (**H,I**). The cross sections of injured muscles from young and old mice were stained for Pax7 (green) and laminin (red). Scale bar = 100 μm. (**D**). Fewer Pax7-positive satellite cells were found in the regenerating area in old mice on DPI 10 compared to young mice (55.1 ±4.3 vs 36.7±5.9 for young vs old, n=4 each, p=0.046) (**D,E**). The cross sections of uninjured muscles from VEGFlo and VEGF WT mice were stained for CD31 (green) and laminin (red). White arrows indicate the presence capillaries (**J**). Despite the decrease in whole muscle VEGFA, capillary density was similar between young and old mice at the ages tested (2.0 ±0.14 vs 2.1±0.15 for young vs old, n=6 each, p=0.923) (**J,K**).

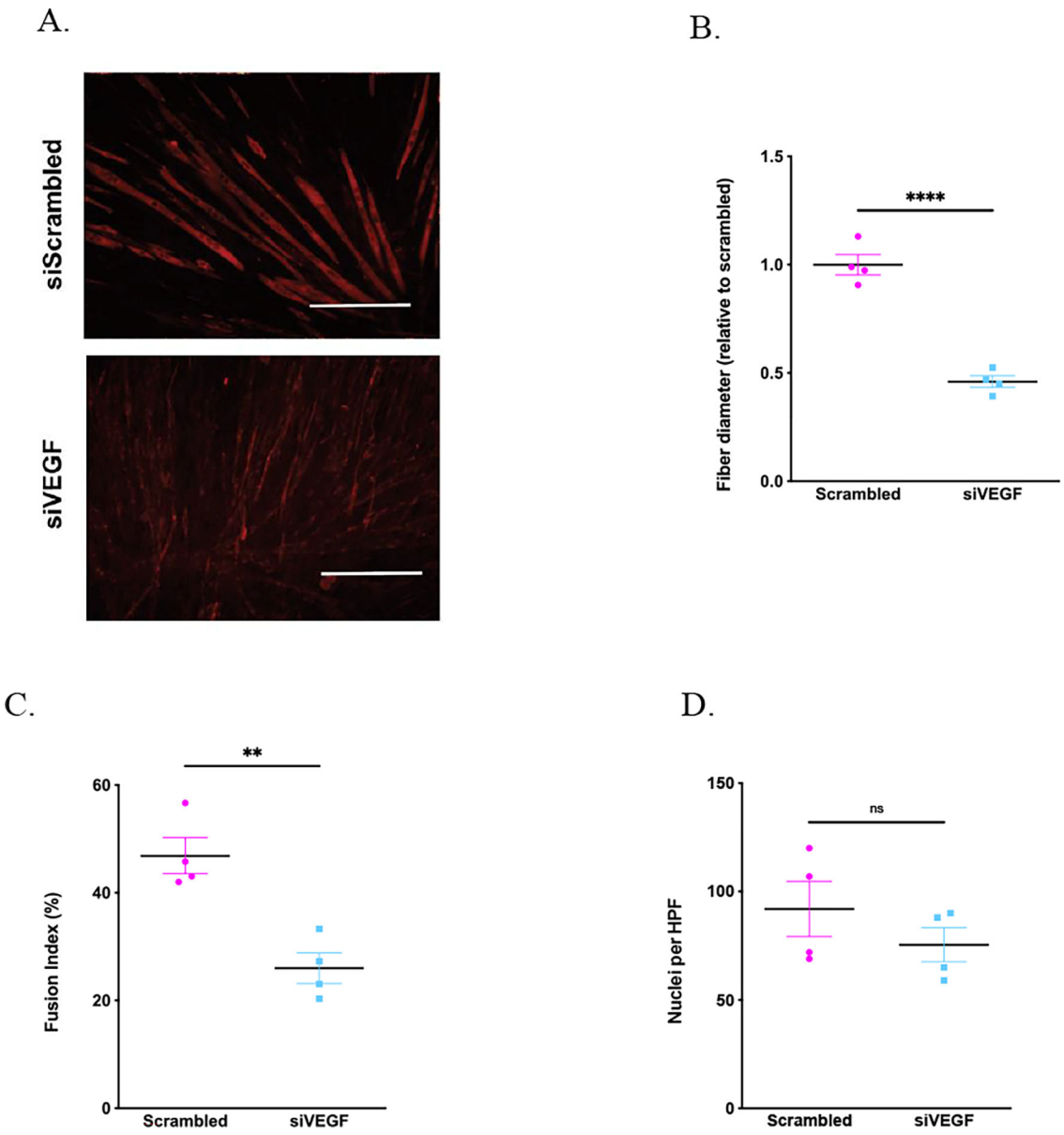

Figure 2. VEFGA inhibition limits skeletal muscle fiber hypertrophy *in vitro*.

C2C12 cells were treated with either scrambled of VEGFA siRNA decreases VEGFA expression by in C2C12 cells. C2C12 cells were stained for MF20 on day 5 differentiation. Scale bar = 100 μm (**A**). VEGFA siRNA reduced muscle fiber diameter (1.00±0.05 vs 0.39±0.03 for scrambled vs VEGF siRNA, n=4 each, p<0.0001) (**A,B**) and fusion index (46.9±3.4 vs 26.0±2.8 % for scrambled vs VEGF siRNA, n=4 each, p=0.003) (**C**) in comparison to scrambled siRNA treatment. The total cell number remained similar in both groups on day 5 differentiation (92.0±12.7 vs 75.5±7.9 for scrambled vs VEGF siRNA, n=4 each, p=0.312) (**D**).

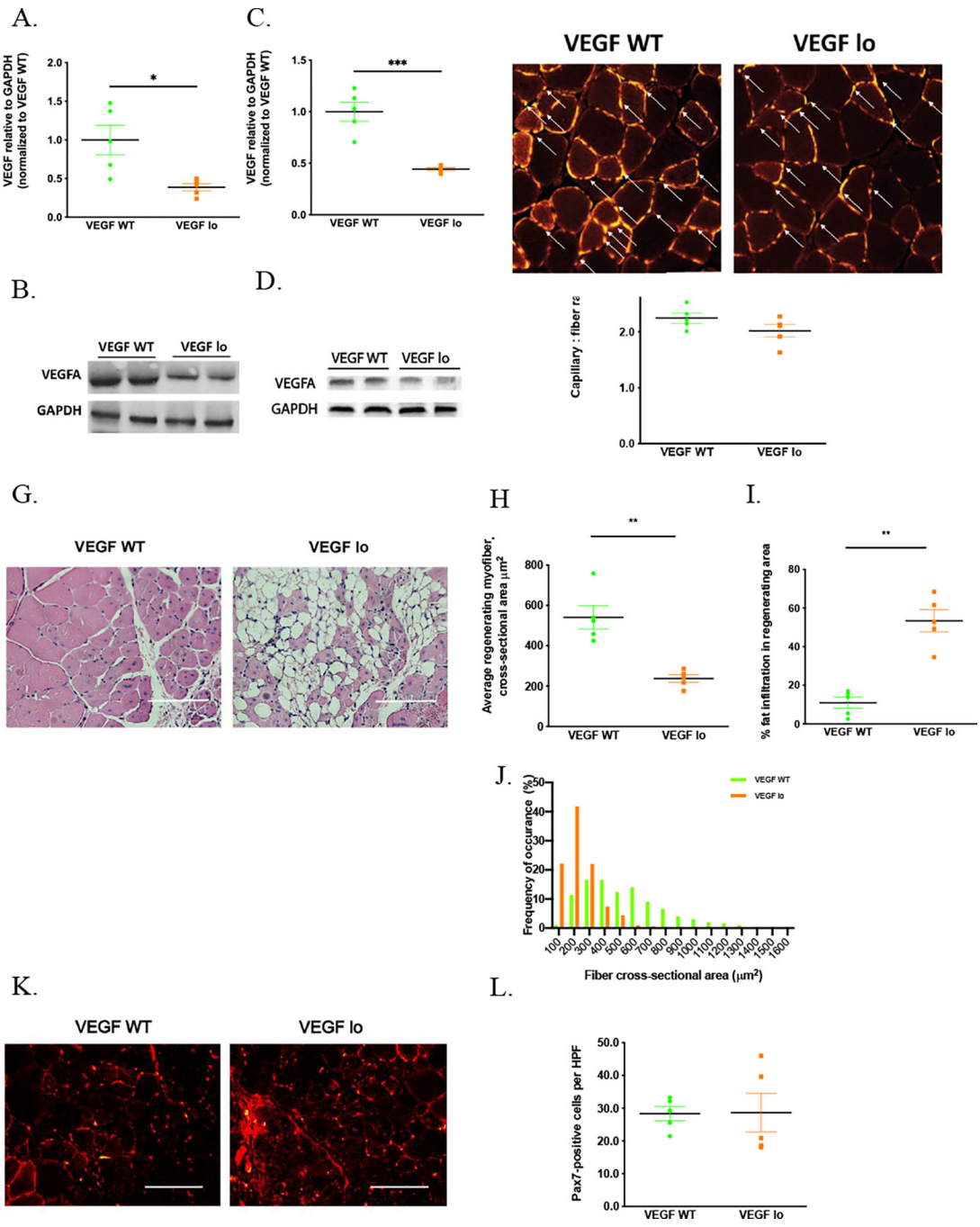

Figure 3. Skeletal muscle regeneration decreases in a transgenic mouse model with reduced VEGFA activity.

To evaluate whether loss of VEGFA impairs myogenic potential, VEGF WT and VEGF^{lo} mice were utilized. Lower protein levels of VEGFA in VEGF^{lo} mice were confirmed by immunoblotting in uninjured (1.00±0.19vs 0.39±0.05 for VEGF WT vs VEGF^{lo}, n=5 each, p=0.01) (**A,B**) and injured muscles 10 days following cryoinjury (1.00±0.09vs 0.44±0.02 for VEGF WT vs VEGF^{lo}, n=5 each, p<0.001) (**C,D**). H&E staining of the cross sections of muscle from the VEGFlo and control mice on DPI 10. Scale bar = 100 µm. (**G**).

Following cryoinjury, the average CSA of regenerating fibers in the VEGF^{lo} mice was significantly reduced on DPI 10 (541.2±5.2 vs 237.9±18.8μm^2 for VEGF WT vs VEGF^{lo}, n=5 each, p=0.001) (**H,J**), in comparison to the littermate controls. Significantly increased fatty deposition was observed after muscle injury in VEGF^{lo} but not littermate control mice (11.0±2.9vs 53.36±5.8 % for VEGF WT vs VEGF^{lo}, n=5 each, p<0.001) (**G, I**). The cross-sections of uninjured muscles from VEGFlo and VEGF WT mice were stained for CD31 (green) and laminin (red). White arrows indicate capillaries. (**E**). The capillary density of uninjured muscle was similar between both VEGF WT and VEGF^{lo} mice in uninjured muscles (2.2±0.09vs 2.0±0.11 for VEGF WT vs VEGF^{lo}, n=5 each, p=0.14) (**E,F**). The cross sections of injured muscles from VEGF WT and VEGF^{lo} mice were stained for Pax7 (green) and laminin (red). Scale bar = 100 m (**K**). Similar numbers of Pax7-positive satellite cells were found in the regenerating area in VEGF^{lo} and control mice on DPI 10 (**D,E**).

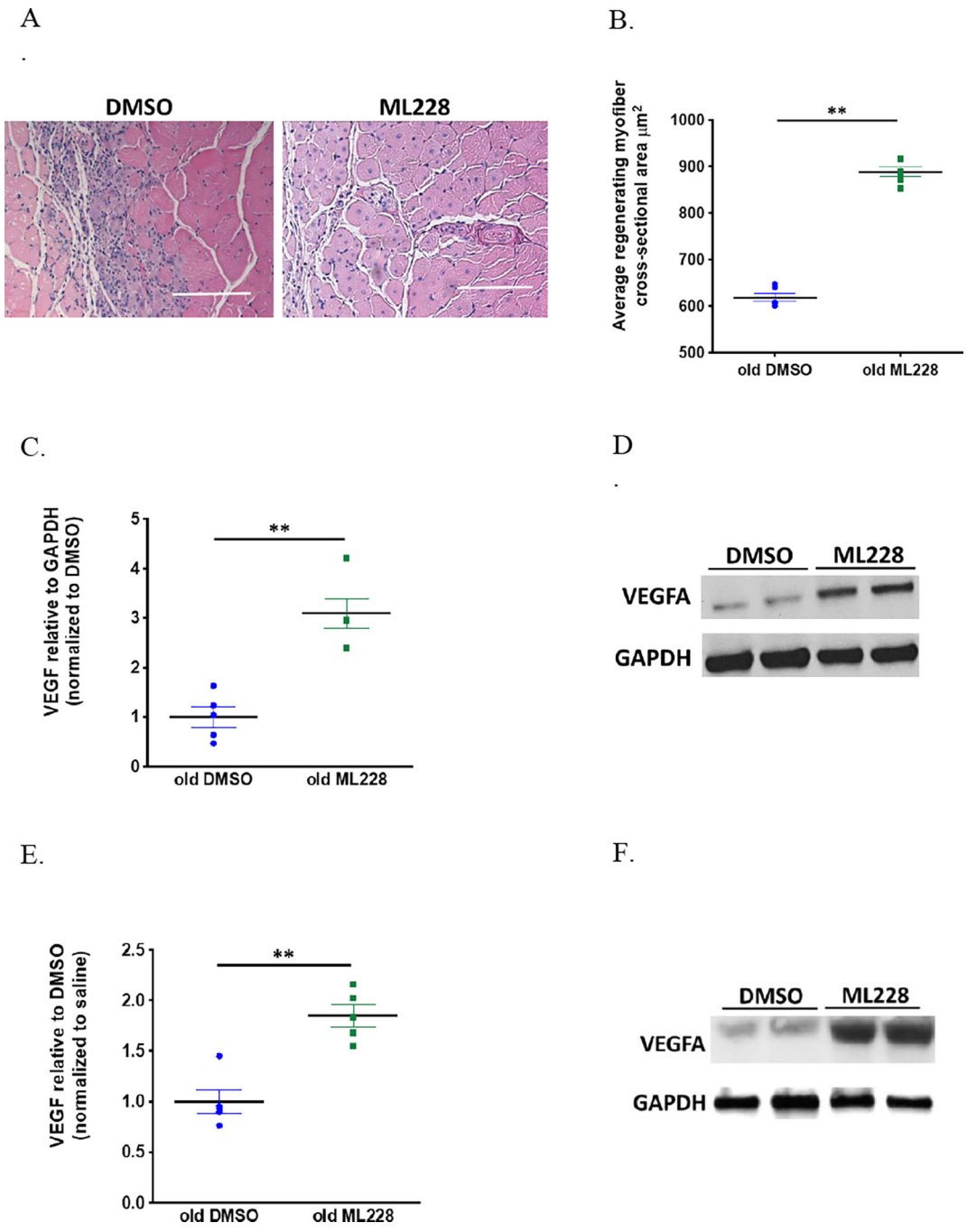

Figure 4. ML228 supplementation increases skeletal muscle VEGF A levels and improves skeletal muscle regeneration in old mice.

Old mice were treated with either ML228 or vehicle control for 5 days. H&E staining of the muscle cross sections from old mice treated with ML228 or DMSO 10 days following cryoinjury. Scale bar = 100 μm (A). Old mice treated with ML228 demonstrated a significant increase in regenerating fiber CSA versus old mice treated with vehicle control (617.9±8.3vs888.5±10.3 μm^2 for old DMSO vs old ML228, n=6 each, $p<0.0001$) (**A,B**). Following ML228 treatment, VEGFA levels increased both at baseline

(1.0±0.2vs888.5±10.3 for old DMSO vs old ML228, n=6 each, p<0.0001) (**C,D**) and 10 days following injury (1.0 ±0.1vs1.8±0.1 for old DMSO vs old ML228, n=6 each, p=0.0008) (**E,F**).

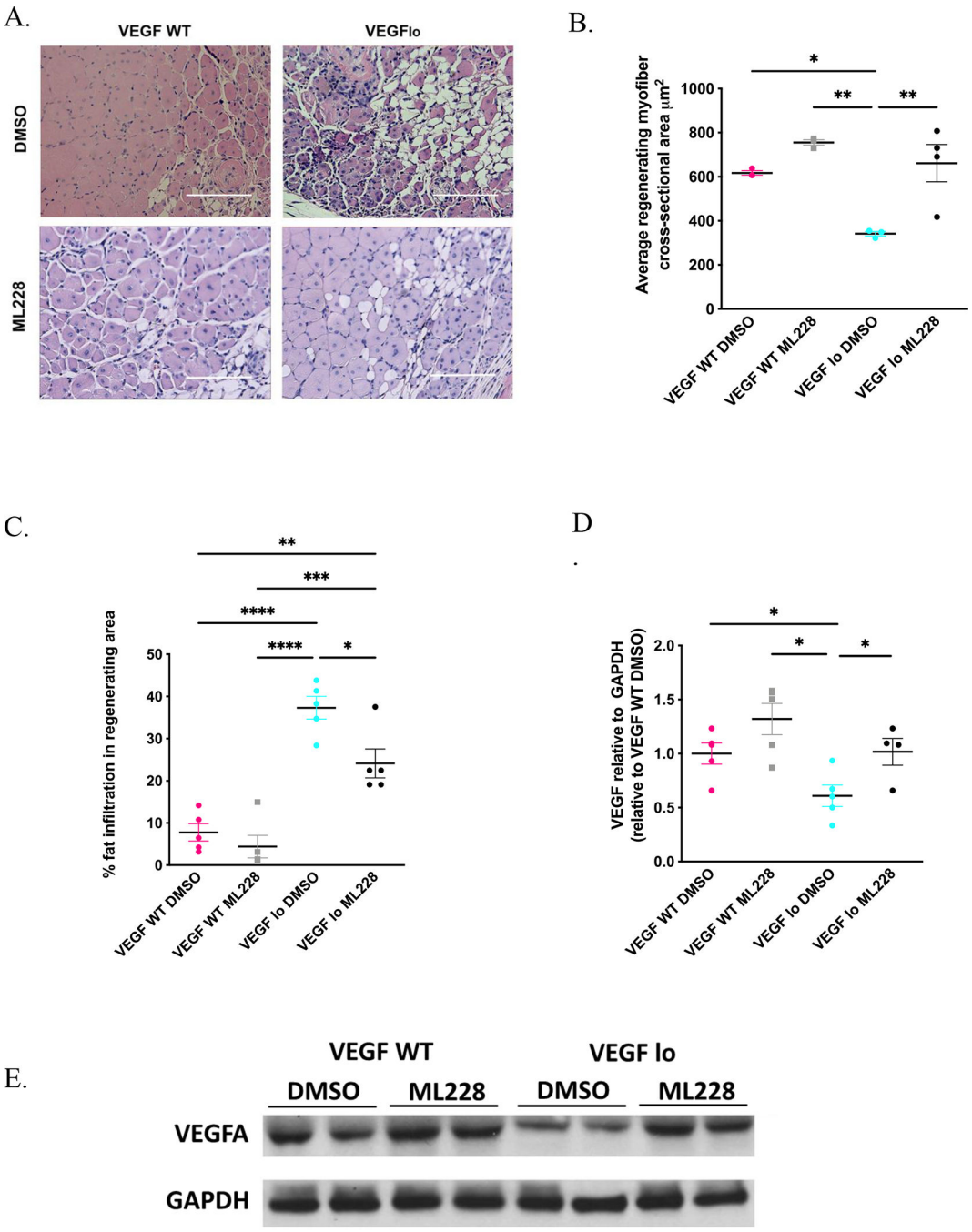

Figure 5. ML228 supplementation improves skeletal muscle regeneration in VEGF^{lo} mice.

H&E staining of cross sections of injured muscles from VEGF^{lo} and control mice 10 days after cryoinjury. Scale bar = 100 µm. (**A**). ML228 supplementation improved skeletal muscle regenerating fiber CSA in VEGF^{lo} in comparison to VEGF^{lo} mice treated with vehicle (341.5±10.4 vs 661.5±4.8 µm^2 for VEGF^{lo} with DMSO vs VEGF^{lo} WT with ML228, n=4 each, p=0.01), but not in VEGF WT mice (617.4±10.3 vs 755.5±12.8 for µm^2 VEGF WT with DMSO vs VEGF WT with ML228, n=4 each, p=0.37) (**A,B**). Fat percentage within the regenerating area also decreased following ML228 treatment in VEGFlo mice (37.3±2.7 vs

24.1±3.4 % for VEGF^{lo} with DMSO vs VEGF^{lo} WT with ML228, n=5 each, p=0.02) (**A,C**). ML228 supplementation also improved VEGFA levels in VEGF^{lo} mice in comparison to vehicle (0.6±0.1 vs 1.0±0.1 % for VEGF^{lo} with DMSO vs VEGF^{lo} WT with ML228, n=4 each, p=0.04), but not in VEGF WT mice (1.0±0.1vs 1.3±0.1 for VEGF WT with DMSO vs VEGF WT with ML228, n=4 each, p=0.10) (**D,E**).