Coordinated Vascular Endothelial Growth Factor Expression and Signaling During Skeletal Myogenic Differentiation

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Angiogenesis is largely controlled by hypoxia-driven transcriptional up-regulation and secretion of vascular endothelial growth factor (VEGF) and its binding to the endothelial cell tyrosine receptor kinases, VEGFR1 and VEGFR2. Recent expression analysis suggests that VEGF is expressed in a cell-specific manner in normoxic adult tissue; however, the transcriptional regulation and role of VEGF in these tissues remains fundamentally unknown. In this report we demonstrate that VEGF is coordinately up-regulated during terminal skeletal muscle differentiation. We reveal that this regulation is mediated in part by MyoD homo- and hetero-dimeric transcriptional mechanisms. Serial deletions of the VEGF promoter elucidated a region containing three tandem CANNTG consensus MyoD sites serving as essential sites of direct interaction for MyoD-mediated up-regulation of VEGF transcription. VEGF-null embryonic stem (ES) cells exhibited reduced myogenic differentiation compared with wild-type ES cells, suggesting that VEGF may serve a role in skeletal muscle differentiation. We demonstrate that VEGFR1 and VEGFR2 are expressed at low levels in myogenic precursor cells and are robustly activated upon VEGF stimulation and that their expression is coordinately regulated during skeletal muscle differentiation. VEGF stimulation of differentiating C2C12 cells promoted myotube hypertrophy and increased myogenic differentiation, whereas addition of sFlt1, a VEGF inhibitor, resulted in myotube hypotrophy and inhibited myogenic differentiation. We further provide evidence indicating VEGF-mediated myogenic marker expression, mitogenic activity, migration, and prosurvival functions may contribute to increased myogenesis. These data suggest a novel mechanism whereby VEGF is coordinately regulated as part of the myogenic differentiation program and serves an autocrine function regulating skeletal myogenesis.

INTRODUCTION

Angiogenesis often coincides with increased vascular permeability, allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells (ECs). Subsequent degradation of the extracellular matrix by matrix-metalloproteases relieves pericyte-EC contacts and liberates extracellular matrix-sequestered growth factors. ECs then proliferate and migrate to their final destination to assemble as lumen-bearing cords. These processes are largely controlled by transcriptional up-regulation and subsequent secretion of three splice variant isoforms of vascular endothelial growth factor (VEGF) and their binding to the endothelial cell tyrosine receptor kinases: VEGFR1 and VEGFR2, leading to activation of a number of downstream signaling cascades (Ferrara *et al.*, 2003).

The function and regulation of VEGF has primarily been studied in pathological angiogenesis, such as in tumors of critical mass where hypoxia-driven HIF1 α activation and

subsequent binding to hypoxia-induced regulatory elements in the VEGF promoter modulates VEGF mRNA expression (Liu *et al.*, 1995; Forsythe *et al.*, 1996; Mazure *et al.*, 1996). Although HIF1 α is known to play a role in the regulation of VEGF gene expression in tissues of the embryo and adult, deletion of the hypoxia-responsive element in the VEGF promoter surprisingly leads to only a subtle defect—adult-onset progressive motor neuron degeneration—with other tissues largely unaffected (Oosthuysen *et al.*, 2001). This observation suggests that the importance of hypoxia-driven HIF1 α regulation of VEGF may be historically overestimated. Moreover, a recent study by Maharaj *et al.* (2006) has shown that VEGF is expressed at appreciable levels in a cell-specific manner in nondiseased normoxic adult tissue. This finding, together with the absence of generalized vascular defects in HIF1 α null mice, suggests that VEGF gene expression in normal adult tissues is controlled by yet to be identified novel mechanisms.

In addition to its induction of new blood vessel growth, VEGF serves an important role in the maintenance and development of endothelial fenestrations (Esser *et al.*, 1998; Lammert *et al.*, 2003; Yokomori *et al.*, 2003) and in endothelial survival in vitro (Darland *et al.*, 2003) and in vivo (Kamba *et al.*, 2006). A growing number of reports suggest that VEGF has autocrine effects on nonvascular cells such as neuronal cells (Ogunshola *et al.*, 2002; Jin *et al.*, 2006; Nishi-

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jima *et al.*, 2007), muscle (Germani *et al.*, 2003), and bone (Fons *et al.*, 2004; Byun *et al.*, 2007). These findings challenge the dogma that VEGF serves solely as a paracrine growth factor specifically targeting ECs (D'Amore, 2007) and may help to explain why VEGF is expressed in normoxic adult tissues, where there is not active ongoing angiogenesis.

In this report, we utilize skeletal muscle as a model system to examine the tissue-specific regulation and function of VEGF under physiological conditions. Although a plethora of data implicates exercise-induced hypoxia as the key signal modulating VEGF expression in the skeletal muscle (Gustafsson and Kraus, 2001; Olfert *et al.*, 2001; Hudlicka *et al.*, 2002), the level of VEGF expression remains high in the skeletal muscle of sedentary mice (Maharaj *et al.*, 2006), suggesting a regulatory mechanism not involving hypoxia. Our data, using multipotent mesenchymal cell lines that are capable of terminally differentiating into skeletal myocytes, demonstrate that skeletal differentiation is associated with increased VEGF expression, suggesting that VEGF is coordinately regulated during myogenesis. We further demonstrate that the myogenic transcription factor, MyoD, and its heterodimeric binding proteins, E12 and E47, up-regulate the expression of endogenous VEGF through direct interaction with the VEGF promoter. Moreover, VEGF-null embryonic stem (ES) cells exhibit a significant reduction in skeletal myogenesis compared with wild-type ES cells, suggesting that VEGF is necessary for skeletal myogenesis. Consistent with these observations, we report that VEGFR1 and VEGFR2 are expressed at low levels in C2C12 cells and are robustly activated upon VEGF stimulation, indicating that VEGF can potentially signal in an autocrine manner. Indeed, VEGF stimulation of C2C12 cells results in increased terminal skeletal muscle differentiation, as evidenced by increased myotube formation, myogenic marker expression, and myotube hypertrophy. Conversely, treatment with the soluble VEGF inhibitor sFlt1 effectively blocks myogenesis and reduces myotube size. These data suggest a novel mechanism by which VEGF expression is coordinately regulated during skeletal myogenesis and demonstrate an autocrine function whereby VEGF serves as an essential regulator in this process.

MATERIALS AND METHODS

LacZ Staining

Adult C57BL/6 mice expressing the β -galactosidase (*lacZ*) reporter gene cDNA with a nuclear localization signal and an internal ribosome entry site inserted into the 3' untranslated region (3'UTR) of the *VEGF* gene were used in these studies (Miquelot *et al.*, 1999). This gene yields a bicistronic mRNA that produces both functional VEGF and a reporter β -galactosidase (β -gal) protein. VEGF expression was visualized in adult skeletal muscle and in cryosections of VEGF-LacZ mouse embryos. Embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). For cryosections, embryos were embedded in OCT compound (Sakura Finetechnical, Torrance, CA). Whole-mounted adult skeletal muscle or cryosections were stained for LacZ using the *in situ* β -galactosidase staining kit, according to the manufacturer's protocol (Stratagene, La Jolla, CA).

Semiquantitative RT-PCR

Skeletal muscle from the hindlimbs of 2-mo-old C57BL/6 mice and lysates from C2C12 and 10T1/2 cultures were collected under RNase-free conditions. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Residual DNA was removed by treatment with 1 U DNase I (Ambion, Austin, TX) at 37°C for 20 min. One microgram of RNA was reverse-transcribed in the presence of 500 ng of oligo dT12–18 with Superscript II reverse transcriptase (Invitrogen) in a 20- μ l reaction at 42°C for 50 min and digested with 2 U of RNase H. One microliter of cDNA was used as a template in a 25- μ l amplification mixture containing 200 mM dNTPs, 1 U *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN), and 0.2 μ M of the appropriate primer pairs capable of amplifying all three isoforms of VEGF (forward: 5' CCT CCG AAA CCA TGA ACT TTC

TGC TC 3'; reverse: 5' CAG CCT GGC TCA CCG CCT TGG CTT 3'). Products were amplified for 35 cycles and separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV light. Quantitation of band intensity was performed using Image J software (<http://rsb.info.nih.gov/ij/>; NIH).

Real-Time PCR

mRNA was purified as described above using the Trizol method. One microgram of RNA was reverse-transcribed as described above, except 500 ng of random hexamers were used. One twentieth of the total cDNA (50 ng of equivalent RNA) was used in each amplification reaction. VEGF isoforms were quantified using the Prism 9700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were performed in 25 μ l with 0.3 μ M primers specific for VEGF isoforms (Zhang *et al.*, 2002) and SYBR Green master mix (ABI, Columbia, MD). PCR cycles consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. To confirm amplification specificity, PCR products from each primer pair were subjected to a melting curve analysis. A standard curve was constructed for each PCR reaction and was derived from the serial dilution (10^{-3} to 10^{-9} ng DNA per reaction) of a plasmid coding for each isoform—VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀—and amplified using the SYBR Green system. The level of isoform expression in each sample was calculated relative to the standard curve. Each sample was run in triplicate, and each experiment included three nontemplate control wells. Results were expressed as the mean $\pm$ SD.

Cell Culture and Treatment

C2C12 (generous gift from Dr. M. T. Chin at Harvard Medical School) and 10T1/2 cells (purchased from ATCC, Manassas, VA; CCL-226) were maintained at subconfluent levels in growth medium (GM) consisting of DMEM (GIBCO, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). 10T1/2 cells, which were used for differentiation experiments, were stably transfected with a MyoD expression plasmid (generous gift from Dr. Mingyao Liu, Texas A&M Health Science Center). To induce differentiation, cells were grown to 100% confluence and GM was replaced with differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (Invitrogen) and 80 U/ml penicillin/streptomycin C. DM was replaced every 24 h.

Bovine retinal endothelial cells (BRECs) were maintained in EBM supplemented with 10% horse serum, 80 U/ml penicillin/streptomycin C, and 12 μ g/ml bovine brain extract. The cells were plated on plastic coated with 50 μ g/ml fibronectin.

Wild-type and VEGF-null mouse ES cells (generous gift from Andras Nagy, Mount Sinai Hospital, Toronto, Canada) were cultured on gelatin-coated dishes in high-glucose DMEM (GIBCO BRL) with 15% fetal bovine serum (Lot FRB25667, Hyclone), sodium pyruvate (GIBCO, stock solution diluted 1:100), nonessential amino acids (GIBCO, stock solution diluted 1:100), β -mercaptoethanol (GIBCO, final concentration 30 μ M), 190 μ g/ml L-glutamine, 60 U/ml penicillin G, 60 μ g/ml streptomycin (glutamine pen-strep mix, Irvine Scientific, Santa Ana, CA), supplemented with media (1:300 dilution) conditioned by Chinese hamster ovary cells overexpressing LIF (provided by Genetics Institute, Cambridge, MA) as a source of LIF to maintain the ES cells in an undifferentiated state. ES cells were cultured in a humidified tissue culture incubator at 10% CO₂ and 37°C and passaged every 2 to 3 d. ES cells were differentiated into cystic embryoid bodies (CEB) as previously described (Ng *et al.*, 2004). Briefly, trypsinized ES cells were suspended in the same culture medium as described above, but without LIF. A total of 60 aliquots (30 μ l) of ES cell suspension containing 2.5×10^3 cells were plated as individual drops onto 100-mm² bacteriological dishes (Valmark, Brampton, Canada). The plates were then inverted and the cells were incubated in hanging drops; this was defined as day 0 of differentiation. The CEB were cultivated via hanging drop for 40–45 h, and then the dishes were turned right side up and 10 ml of ES culture media without LIF was added so that the CEB were then in suspension. Every 3 d, half of the culture media was removed and replaced with fresh media. For attached cultures, day 4 or day 5 CEB were transferred to gelatin-coated glass coverslips, onto which the CEB attached, flattened, and spread.

Recombinant human VEGF₁₆₅ (obtained from National Cancer Institute, www.cancer.gov) was added to the cultures at a final concentration of 25 ng/ml. Recombinant mouse VEGF-R1/Fc chimera (sFlt1; R&D Systems, Minneapolis, MN; 471-F1, Lot BSL1107011) was added to the cultures at a final concentration of 100 ng/ml. Recombinant adenovirus (AV) vectors containing either a null cassette or sFlt1 were obtained from Qbiogene (Montreal, Canada). Undifferentiated C2C12 cells were incubated with AV vectors at multiplicities of infection (MOIs) of 10^3 viral genomes per cell.

Western Blotting

Tissues were collected in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM Na₃VO₄, and 20 mg/ml aprotinin). Proteins were quantified using the D_c protein assay kit (Bio-Rad, Richmond, CA). Cell extracts were analyzed by SDS-PAGE and probed with anti-MyoD (sc-304,

Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGFR1 (sc-316, Santa Cruz Biotechnology), anti-VEGFR2 (a generous gift from A. Kazlauskas, Schepens Eye Research Institute), anti-myosin heavy chain (MHC; MF20, Iowa Hybridoma Bank), anti-myogenin (F5D, Iowa Hybridoma Bank), PY20 (05-321, Upstate Biotechnology, Lake Placid, NY), and anti-tubulin (CP06-100UG, Oncogene Research Products, Boston, MA). Binding was detected with the appropriate HRP-conjugated secondary antibody (mouse: NA931V, rabbit: NA934V, Amersham Biosciences, Piscataway, NJ) and ECL-Plus Western Blotting Detection System (RPN2132, GE Healthcare, Waukesha, WI).

Immunofluorescence

Cells were fixed in ice-cold methanol for 10 min. Reactions were blocked for 60 min with PBS containing 0.2% bovine serum albumin, followed by 60 min of incubation with anti-MHC antibody (1:20 dilution; Iowa Hybridoma Bank). Fluorescein-conjugated goat anti-mouse antibody was added for 30 min (1:1000; 81-6511, Molecular Probes, Eugene, OR). Nuclear staining was observed after 10 min of 4',6-diamidino-2-phenylindole (DAPI) treatment (1:100 dilution; Molecular Probes). Fluorescence images were captured on a CCD camera mounted on an inverted research microscope using Spot imaging software (Diagnostic Instruments, Sterling Heights, MI).

Luciferase Reporter Assays

VEGF-promoter luciferase constructs have been previously described (Loureiro *et al.*, 2005). C2C12 and 10T1/2 cells were cotransfected at 60–80% confluence using Lipofectamine 2000 with equal amounts of VEGF firefly luciferase (Fluc)-reporter plasmid, *Renilla* control luciferase (Rluc; Promega, Madison, WI), and the indicated plasmid to a total of 1.5 μ g DNA per well of a 24-well plate. Fresh medium was added 6 h after transfection and left undisturbed until cell lysates were collected after 48-h incubation. Luciferase expression was detected using the Dual Luciferase Reporter Assay System (Promega) following the manufacturer's instructions with a Turner Lumimometer (Turner Designs, Sunnyvale, CA). VEGF promoter activity, as detected by Fluc activity, was normalized against Rluc readings and this ratio was divided by the ratio of readings from promoterless luciferase to determine fold-change. Assays were performed three times in triplicate with similar results; one representative experiment is shown. Statistical analysis of the data were performed to determine significance using analysis of variance.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay (ChIP) was performed following the protocol outlined by the ChIP assay kit (Upstate Biotechnology). Briefly, C2C12 cells were fixed with 1% formaldehyde, scraped into conical tubes, pelleted, and lysed in SDS lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin A. DNA was sheared to fragments of 200–500 base pairs by eight 10-s sonications. The chromatin was precleared with salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology) for 1 h at 4°C with gentle agitation. The agarose beads were pelleted, and the precleared supernatant was incubated with antibodies to IgG or MyoD overnight at 4°C. The region between –256 to –122 bases upstream of the VEGF 5'UTR was PCR amplified from the immunoprecipitated chromatin using the appropriate primers (Forward: 5'-CACTCTCCTGTCTCCCCTGA-3'; Reverse: 5'-CACTACCGCGAAATGGAAG-3'). After PCR, the PCR product was resolved on a 2.5% agarose gel and stained with ethidium bromide. Samples were visualized under UV light.

Invasion/Migration Assay

C2C12 cells were seeded on six-well plates and grown to 100% confluence in DMEM + 10% FBS (serum starvation was not used in this experiment because low serum forced the cells to differentiate and inhibited migration almost completely) and wounded with a sterile pipette tip to remove cells by two perpendicular linear scratches. After washing, the cells were cultured with DMEM + 10% FBS under the following conditions: mock treatment, 25 ng/ml VEGF, or 100 ng/ml sFlt1. The progress of migration was photographed immediately after injury and at 12 h after wounding, near the crossing point, with an inverted microscope equipped with a digital camera (SPOT; Diagnostic Imaging, Sterling Heights, MI).

Proliferation Assay

Proliferation assays were performed according to the method of Lyons (2000). Briefly, C2C12 cells were resuspended at 10^6 cells/ml in PBS, and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDASE) was added to a final concentration of 5 μ M and incubated at 37°C for 10 min. The cells were then washed two times with DMEM media supplemented with 10% FBS, plated in six-well plates, and allowed to proliferate for 48 h in DMEM + 10% FBS in the presence of control or 100 ng/ml sFlt1. Cells were collected by trypsinization and washed two times in PBS, and fluorescence was read using a Becton Dickinson FacsSort flow cytometer (Mountain View, CA).

Apoptosis Assay

Apoptosis was measured using the annexin V binding method (Koopman *et al.*, 1994). Briefly, C2C12 cells at day 2 of differentiation conditions were treated with control or 100 ng/ml sFlt1, and cells were washed once with PBS, trypsinized, and resuspended briefly in serum containing medium to quench the trypsin. Cells were washed once in PBS, and 1×10^5 cells were added to annexin V binding reagent for 15 min in the dark. Propidium iodide was included in the reaction to allow the identification of cells in late apoptosis. Fluorescence was read using a Becton Dickinson FacsSort flow cytometer.

RESULTS

Expression of VEGF in the Developing and Adult Skeletal Muscle

To investigate VEGF expression in the mouse embryo and adult skeletal muscle, representative sections from VEGF-lacZ E18.5 mouse limbs and adult skeletal muscle whole mounts were stained with X-gal. Robust LacZ staining was observed in both the embryonic and adult time points (Figure 1A). Although a high level of VEGF expression is not surprising in embryonic samples, with the exception of exercise-induced hypoxia, little published data exists regarding the physiological expression and control of VEGF in the adult skeletal muscle. Our data are consistent with a report published by Maharaj *et al.*, (2006), indicating strong expression of VEGF in adult mouse skeletal muscle.

The VEGF gene encodes for at least three biochemically distinct protein isoforms generated through alternative splicing: VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈ (Tischer *et al.*, 1991; Shima *et al.*, 1996). These different gene products exhibit tissue-specific expression during embryogenesis (Ng *et al.*, 2001) and in the adult (Bacic *et al.*, 1995; Ng *et al.*, 2001; Maharaj *et al.*, 2006). To examine VEGF isoform levels in adult skeletal muscle, we collected skeletal muscle from the hindlimb of 2-mo-old mice and performed semiquantitative RT-PCR using primers that specifically amplify each VEGF isoform. Previous observations indicate a good correlation between VEGF mRNA and protein levels and give strong reason to believe that the mRNA levels are an accurate reflection of protein levels (Shima *et al.*, 1995; Cheng *et al.*, 1997). As seen in Figure 1B, VEGF₁₆₄ and VEGF₁₈₈ were the only detectable isoforms expressed in adult mouse skeletal muscle tissue. VEGF₁₆₄ and VEGF₁₈₈ are known to bind to heparan sulfate within the extracellular matrix (Park *et al.*, 1993; Ballaun *et al.*, 1995), suggesting an autocrine or paracrine action as the primary means of VEGF signaling in skeletal muscle. We confirmed these results using real time PCR using primers that specifically amplify each VEGF isoform. Indeed, our results confirmed that VEGF₁₆₄ and VEGF₁₈₈ are the predominant isoforms in adult skeletal muscle, with no detectable levels of VEGF₁₂₀ (Figure 1C).

VEGF Is Up-Regulated during Myogenic Differentiation

We next sought to elucidate the molecular mechanism controlling VEGF expression in skeletal muscle. We utilized in vitro cell culture models that closely recapitulate the formation and maintenance of skeletal muscle. Two cell lines serve as excellent model systems to examine the molecular mechanisms controlling skeletal muscle differentiation: the multipotent mesenchymal progenitor cell line C2C12 (Bains *et al.*, 1984) and the fibroblast cell line 10T1/2 (Davis *et al.*, 1987). To determine if VEGF is endogenously expressed in these cell lines, we performed semiquantitative RT-PCR to detect the VEGF isoform expression. As demonstrated in Figure 2A, VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈ were all expressed in both cell lines, indicating that these lines likely serve as good model systems to examine the mechanisms controlling VEGF gene expression. It is worth noting that

Figure 1. VEGF is expressed in embryonic and adult skeletal muscle. (A) Cryosections of embryonic mouse limbs (E18.5) and whole mounts of adult mouse skeletal muscle were collected from VEGF-LacZ mice and stained for LacZ using in situ β -galactosidase. Scale bar (E18.5) 50 μ m; scale bar (adult) 100 μ m. Support of mRNA was collected from the hindlimb skeletal muscle of 2-mo-old C57BL/6 mice, and semiquantitative RT-PCR detection of VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀ was performed. As a reaction control, a PCR reaction composed of mixed VEGF plasmids each encoding single VEGF isoforms was utilized. (C) mRNA was collected from the hindlimb skeletal muscle of 2-mo-old C57BL/6 mice and real-time RT-PCR detection of VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀ was performed. The relative amount of each VEGF isoform is represented as a percentage of the total VEGF expression (VEGF₁₈₈ + VEGF₁₆₄ + VEGF₁₂₀ = 100%). Error bars, SDs.

VEGF was expressed at higher levels in C2C12 cells compared with 10T1/2 cells.

On reaching confluence and serum withdrawal, C2C12 cells undergo differentiation from proliferative myoblasts to terminally differentiated myotubes, fusing together to form multinucleated cells that express markers of differentiated skeletal muscle (Figure 2B and C). 10T1/2 cells, when exogenously overexpressing the myogenic transcription factor MyoD, have also been demonstrated to undergo terminal differentiation similar to C2C12 cells (Pinney *et al.*, 1988). To better understand the expression of VEGF during skeletal muscle differentiation, we induced C2C12 and 10T1/2 cells to undergo terminal differentiation and performed semiquantitative RT-PCR on samples taken in the proliferative state and throughout the process of differentiation. Within

1 d of differentiation conditions, the levels of all VEGF isoforms were increased in both 10T1/2 (Figure 2D and E) and C2C12 (Figure 2F and G) cells; levels returned to baseline levels after 5 d of differentiation. These data suggest that VEGF expression is coordinately controlled with the process of myogenic differentiation.

The Myogenic Transcription Factor MyoD Controls VEGF Transcriptional Expression

Given the unique expression patterns of the VEGF isoforms during skeletal myogenesis, we sought to determine the molecular mechanisms controlling this process. Using TESS analysis (<http://www.cbil.upenn.edu/cgi-bin/teess/teess>) of the human VEGF promoter, we identified six putative CANNTG sites within the promoter region, 1600 bases upstream of the coding region (Figure 3A). MyoD homodimers or heterodimers of MyoD plus E12 or E47 serve as transcription factor complexes that bind to CANNTG consensus sites in the promoter regions of genes, performing major functions in specification and differentiation of skeletal muscle precursor cells (Murre *et al.*, 1989).

These predictions led us to test whether MyoD regulates VEGF expression during the muscle differentiation program. In C2C12 cells we stably overexpressed a control vector, a wild-type MyoD, or a dominant negative MyoD (dnMyoD) in which MyoD is fused to the lysosomal protease cathepsin B, thus proteolytically digesting any MyoD multimers and/or detour the multimers from their usual subcellular destination to the lysosome (Li *et al.*, 1996). We then used semiquantitative RT-PCR to examine the endogenous VEGF expression levels in each condition. Overexpression of wild-type MyoD led to a significant increase in VEGF isoform levels over the control condition, whereas dnMyoD expression resulted in a marked reduction in VEGF isoform expression compared with the control (Figure 3B and C). These data suggest that MyoD is necessary to maintain a baseline level of VEGF transcriptional expression in C2C12. This is consistent with our findings that C2C12 cells, which expressed endogenous MyoD, synthesize higher levels of VEGF than 10T1/2 cells, which lack endogenous MyoD (Figure 2A).

To confirm our finding that MyoD modulates VEGF expression in skeletal muscle precursor cells, we utilized luciferase reporter assays. C2C12 cells were transfected with the 9-kb (full length) VEGF promoter luciferase reporter plasmid and combinations of 1) control vector, 2) wild-type MyoD, 3) E12, or 4) E47, and luciferase assays were performed on the cell lysates. As observed in Figure 3D, coexpression of the VEGF luciferase plasmid with MyoD led to an approximately fourfold increase in VEGF-reporter expression over the control. Transfection with either E12 or E47 alone resulted in no increase in VEGF-reporter expression; however, cotransfection of either MyoD and E12 or MyoD and E47 led to eightfold increase in luciferase activity over the control. These data suggest that MyoD and the E-box proteins act synergistically to up-regulate VEGF-reporter expression.

We next set out to determine the specific transcriptional binding sites that mediate the MyoD-dependent increase of VEGF expression. C2C12 cells were transfected with truncation mutants of the VEGF promoter luciferase reporter plasmid and either a control plasmid or wild-type MyoD. Strong MyoD-driven VEGF-reporter expression was observed in full-length promoter constructs and truncations down to -160 bases upstream of the transcriptional start site (Figure 3E). However, truncations less than this region led to complete loss of MyoD-mediated transcriptional expression of

Figure 2. VEGF expression is regulated during skeletal myogenic differentiation. (A) Semiquantitative RT-PCR for VEGF isoform and MyoD expression was performed on cell lysates of C2C12 and 10T1/2 cells. Steady-state mRNA levels were detected and GAPDH expression was utilized to indicate equal loading. (B) Phase-contrast images of C2C12 cells were taken in the proliferative phase (GM) and after 3 d of differentiation (DM). (Arrows indicate the presence of myotubes after 3-d differentiation, 40 $\times$ magnification) (C) Western blot analysis of the muscle-lineage-specific marker MyoD was performed on lysates collected from proliferating (GM) and 3-d differentiated (DM) C2C12 cells. Tubulin expression was utilized to indicate equal loading. (D–G) RNA was collected from C2C12 and 10T1/2 cells in the proliferative phase of growth (GM) and over 4 d of differentiation (DM1–4). Semiquantitative RT-PCR for VEGF isoform expression was performed for each time point for C2C12 (D) and 10T1/2 (F). Analysis of GAPDH expression was utilized as a loading control. Quantification of VEGF isoform expression during C2C12 (E) and 10T1/2 (G) differentiation was performed using NIH ImageJ software.

the reporter gene, suggesting that a region between -247 bases and -160 bases upstream of the transcription start site is essential for MyoD activity on this promoter. Indeed, within this region lies three closely spaced CANNTG sites at -165 , -187 , and -198 bases upstream of the VEGF 5' UTR.

MyoD Physically Associates with the VEGF-Promoter

To demonstrate that MyoD directly interacts and forms a complex with the MyoD sites in this region, we performed ChIP using sheared DNA isolated from C2C12 cells. Immunoprecipitation of chromatin-bound DNA using an antibody against endogenous MyoD was followed by PCR using primers that amplified the -256 - to -122 -base pair region of the VEGF promoter spanning the length of the three essential consensus MyoD sites. As demonstrated in Figure 3F, the MyoD-specific antibody is capable of immunoprecipitating the VEGF promoter fragment containing the three MyoD sites, whereas control immunoprecipitation using anti-IgG failed to produce a PCR product.

VEGF-Null Embryonic Stem Cells Exhibit Decreased Skeletal Myogenesis

The essential role of VEGF during vasculogenesis and angiogenesis is well established (Ferrara, 1999); however, very little is known regarding nonvascular roles for VEGF. To determine if VEGF influences skeletal myogenic differentiation, we utilized a CEB model in which ECs are cultivated to form aggregates, which efficiently differentiate into a number of cell lineages, including skeletal muscle. This sys-

tem closely recapitulates the early steps of muscle development in vivo (Rohwedel *et al.*, 1994) and serves as an excellent in vitro system to study this process. ES cells isolated from wild-type or VEGF-null mice were differentiated into CEB and then transferred to gelatin-coated glass coverslips, where they attached, flattened, spread, and differentiated. Over a period of 11 d, we performed immunofluorescent staining specific for the skeletal muscle specific marker, MHC, and calculated the percentage of each field that was positive for MHC. Myosin heavy chain was first detected at day 7 in both the wild-type and VEGF-null ES cultures in sparse, isolated patches throughout the CEB. At this time point staining in wild-type cultures represented $\sim 3.5\%$ of the total area of the representative fields, whereas MHC staining in VEGF-null cells represented $\sim 0.3\%$ of the total area of the representative fields (Figure 4A and B). At days 9 and 11 of differentiation, VEGF-null ES cultures consistently demonstrated a reduction in MHC expression compared with CEB derived from wild-type ES cells. Because the MHC antibody (Iowa Hybridoma Bank, MF20) we used in these experiments is known to react with any striated muscle actin, including cardiac tissue, we performed Western blots on wild-type and VEGF-null ES cell lysates at day 9 of differentiation to detect the expression of the skeletal muscle specific protein, myogenin. As expected, a significant reduction in myogenin protein expression is observed in VEGF-null ES cell lysates compared with wild-type ES cell lysates (Figure 4C). These data demonstrate that while the loss of VEGF does not appear to affect the onset of myogenic

Figure 3. The myogenic transcription factor MyoD regulates the expression of VEGF. (A) Illustration depicting consensus CANNTG MyoD binding sites located within the first 1600 bases upstream of the VEGF 5' UTR. (B and C) Semiquantitative RT-PCR for VEGF isoform expression was performed on cDNA collected from C2C12 cells stably overexpressing an empty plasmid (control), wild-type MyoD (MyoD), or dominant negative MyoD (dnMyoD). GAPDH detection was utilized as a loading control. Quantification of VEGF isoform expression was performed using NIH ImageJ software. (D) 10T1/2 cells were transiently transfected with the 9-kb full-length VEGF promoter region driving expression of the firefly luciferase gene in combination with empty vector, wild-type MyoD, E12, and/or E47. A *Renilla* luciferase vector was included in all conditions as a transfection control. Firefly luciferase activity was measured and equalized to the control *Renilla* luciferase activity. Luciferase activity is expressed as arbitrary luciferase units. Each experiment was performed in triplicate and repeated at least three times. Data are reported as the mean $\pm$ SD for each representative experiment. (E) 10T1/2 cells were transiently transfected with the indicated truncation constructs of the VEGF promoter region driving expression of the firefly luciferase gene and either a control vector or wild-type MyoD and normalized with *Renilla* luciferase as above. Data are reported as the percentage of increase in MyoD-transfected cells over control-transfected cells with mean $\pm$ SD for each representative experiment. (F) Chromatin immunoprecipitation analysis of C2C12 lysates detecting direct MyoD binding to the VEGF promoter region between -256 to -122 bases upstream of the VEGF 5' UTR. IgG incubation was utilized as a control.

differentiation, VEGF is necessary for this process to proceed in a robust manner. However, these findings do not demonstrate whether myogenic cells are responding to their own VEGF production or from VEGF secreted in a paracrine manner from other cell types in the culture.

VEGF Receptor Expression and Activation in C2C12 Cells

Although VEGF was initially reported to be an endothelial-specific growth factor (Ferrara and Davis-Smyth, 1997), recent reports have indicated the expression of VEGF receptors in a variety of non-ECs (Ishida *et al.*, 2001; Olfert *et al.*, 2001; Ishii *et al.*, 2002; Rissanen *et al.*, 2002; Germani *et al.*, 2003; Gustafsson *et al.*, 2005; Wilgus *et al.*, 2005; Maharaj *et al.*, 2006; Onofri *et al.*, 2006; D'Amore, 2007). To compare the expression of VEGFR1 and VEGFR2 in C2C12 cells with cultured ECs, which are known to express high levels of these two receptors (Rajah and Grammas, 2002), we performed Western blot analysis on lysates collected from BRECs and C2C12 cells. Our analysis demonstrated a strong expression of both

VEGFR1 and VEGFR2 in BRECs, whereas levels of both VEGFR1 and VEGFR2 in C2C12 cells were significantly less (Figure 5A). Although the level of these receptors in C2C12 cells is lower relative to ECs, this finding suggests that VEGF signaling may play a role in C2C12 cells. To determine if the VEGF receptors are phosphorylated and become active upon VEGF stimulation of C2C12 cells, we collected cell lysates from C2C12 cells stimulated for 2 min in the presence of either 2% horse serum (as a control) or 2% horse serum plus 25 ng/ml VEGF. Immunoprecipitation of these lysates using an antibody specific for phosphotyrosine (anti-PY20), and subsequent Western analysis with anti-VEGFR2 antibodies revealed a significant increase in VEGFR2 activation (Figure 5B), suggesting that VEGF rapidly induces signaling in C2C12 cells.

Although we observed VEGF receptor expression in C2C12 cells and demonstrated their activation after VEGF treatment, it is important to determine if the VEGF receptors are expressed at appreciable levels during the process of

Figure 4. VEGF regulates skeletal myogenesis during embryonic stem cell differentiation. (A) ES cells were differentiated using the hanging drop method as described in *Materials and Methods*. At 7, 9, and 11 d of differentiation, cells were fixed and MHC was detected by immunofluorescence (green) and DAPI (blue) was performed (40 $\times$ magnification). (B) Quantification of MHC positive area within the field was performed using NIH ImageJ software. Data are reported as the mean $\pm$ SD for each representative experiment. (C) Lysates from ES cells were collected at day 9 of differentiation and Western-blotted with anti-myogenin and anti-tubulin antibodies.

skeletal myogenesis. To address this, we collected cell lysates from C2C12 cultures in their proliferative phase and throughout myogenic differentiation. Western blot analysis revealed an inverse relationship between the expression of VEGF receptors and differentiation (Figure 5C); although VEGFR1 expression was highest in the proliferative state and gradually decreased during myogenic differentiation, VEGFR2 expression was relatively low in the proliferative state and significantly increased during differentiation. These data suggest that the induction of VEGF expression during skeletal myogenesis via MyoD-dependent up-regulation may induce an autocrine signaling pathway that performs a necessary role in myogenic differentiation.

Figure 5. VEGF receptor expression and activation during skeletal myogenesis. (A) Lysates from BREC and C2C12 cells were Western blotted for VEGFR1 and VEGFR2. Tubulin levels were utilized as a loading control. (B) Lysates from C2C12 cells grown in 2% horse serum with (VEGF) or without (control) the 25 ng/ml VEGF (2 min) were immunoprecipitated with anti-VEGFR2 antibodies and Western blotted with anti-PY20 and anti-VEGFR2 antibodies were performed to detect VEGFR2 phosphorylation. (C) Lysates from C2C12 cells in proliferative conditions (GM) and days 1 through 4 of myogenic differentiation (DM1-4) were subjected to Western analysis to detect levels of VEGFR1 and VEGFR2. Detection of MHC levels demonstrated myogenic differentiation and tubulin protein levels were used as a loading control.

VEGF Stimulation of C2C12 Cells Enhances Myogenic Differentiation

Given that C2C12 cells provide a well-documented model in which to examine the effects of VEGF on myogenesis, we utilized phase-contrast microscopy to quantify myotube formation during C2C12 myogenic differentiation in response to VEGF stimulation or inhibition. As observed in Figure 6A and C, the addition of 25 ng/ml VEGF to differentiating C2C12 cells resulted in an $\sim 30\%$ increase in the number of myotubes compared with untreated control cells. Moreover, inhibition of autocrine VEGF signaling via the expression of sFlt1 led to an $\sim 70\%$ reduction in myotube numbers relative to AV-control (Figure 6A and C). We confirmed these results using fluorescent detection of the skeletal muscle-specific differentiation marker, MHC. Similar to our findings measuring myotube numbers, addition of VEGF led to an increase in MHC-positive cells compared with cells that received control treatment (Figure 6B), whereas inhibition of autocrine VEGF signaling using sFlt1 resulted in decreased MHC-positive cells relative to controls (Figure 6B). Additionally, VEGF stimulation of differentiating C2C12 cells resulted in a significant increase in myotube hypertrophy, although sFlt1 inhibition of VEGF signaling led to a decrease

Figure 6. VEGF regulates C2C12 skeletal myogenic differentiation. (A and B) C2C12 cells were differentiated for 3 d in the following conditions: control, VEGF, AV-control, or AV-sFlt1. Phase-contrast images (40 $\times$ magnification; A) and immunofluorescent detection of MHC (MHC; green) and DAPI (blue; 100 $\times$ magnification; B) were collected at day 3 of differentiation. (C) Quantification of myotube number per field was performed using NIH ImageJ software. Data are reported as the mean $\pm$ SD for each representative experiment. (D) Quantification of relative MHC-positive cell size using NIH Image J software (red dashed line indicates median size of MHC positive control or AV-control cells).

in myotube size compared with controls (Figure 6D). These results strongly suggest that VEGF signals to C2C12 cells through an autocrine mechanism.

VEGF Regulates C2C12 Cell Fate Decisions

To determine the mechanism by which VEGF promotes myogenic differentiation, we examined its effect on MHC expression in C2C12 cells. At day 3 of myogenic differentiation, levels of MHC were up-regulated in VEGF-treated cells compared with controls (Figure 7A), revealing that VEGF is capable of promoting skeletal myogenic differentiation by up-regulating key proteins involved in muscle function. Moreover, VEGF neutralization resulted in a significant down-regulation of MHC expression compared with controls, suggesting that VEGF is essential for complete myogenic differentiation.

Myoblast migration is central to both developmental and regenerative myogenesis (Schultz and McCormick, 1994; Christ and Brand-Saberi, 2002), in which differentiation, migration, and cell-to-cell contacts are coupled (O'Connor *et al.*, 2007). Given that VEGF has been shown to induce cell migration in a number of cell types (Gruber *et al.*, 1995; Ratajska *et al.*, 1995; Yoshida *et al.*, 1996; Grosskreutz *et al.*, 1999; Castellon *et al.*, 2002), it seemed likely that it would perform a similar function during myoblast migration. To test this possibility, we performed invasion/migration as-

says on C2C12 cells treated with 25 ng/ml VEGF, 100 ng/ml recombinant sFlt1, or with untreated cells as a control. Confluent monolayers of cells were physically wounded with a scratch and allowed to migrate in order to "heal" for 12 h, eliminating the possibility of proliferation accounting for the wound closure. As demonstrated in Figure 7B, at 12 h after the initial scratch VEGF-treated cells had completely closed the wound, whereas sFlt1-treated cells exhibited a significant reduction in invasion/migration compared with the control. These data suggest that VEGF stimulates C2C12 cell migration and that endogenous VEGF plays an essential role in C2C12 invasion/migration.

In addition to its action on the endothelium, VEGF has been heavily implicated as a survival factor for nonvascular cells (Zachary, 2001; Darland *et al.*, 2003; Nishijima *et al.*, 2007). To determine if the promyogenic effect of VEGF signaling might occur through its anti-apoptotic signaling, differentiated C2C12 cells were treated with 100 ng/ml recombinant sFlt1 for 48 h and examined for apoptosis via Annexin V:propidium iodide staining and subsequent flow cytometric analysis. As demonstrated in Figure 7C, sFlt1-treated cells exhibited an $\sim$ 50% increase in the number of apoptotic cells compared with control cells, suggesting that VEGF signaling is essential for cell survival during C2C12 differentiation, an effect that may contribute to the promyogenic effects of VEGF.

Figure 7. VEGF serves multiple roles in C2C12 cells. (A) Lysates were collected from C2C12 cells grown at 3 d of differentiation and treated with 25 ng/ml VEGF, 100 ng/ml sFlt1, or left untreated and subsequently subjected to Western analysis to detect steady-state levels of MHC. Tubulin was utilized as a loading control. (B) C2C12 cells were grown to 100% confluence, treated as in A and wounded with a sterile pipette tip to remove cells. Photographs were taken ($40\times$ magnification) at $t = 0$ and $t = 12$ h (control, VEGF, and sFlt1) after the injury. (C) Fluorescence-activated cell sorter analysis for annexin V:propidium iodide staining of C2C12 cells at day 2 of differentiation and treated 100 ng/ml sFlt1. (D) Flow cytometric analysis of CFDASE stability in proliferating C2C12 cells treated with 25 ng/ml VEGF or 100 ng/ml sFlt1 (red dashed line indicates median fluorescence of control cells).

Muscles are formed by fusion of individual postmitotic myoblasts to form multinucleated myotubes; however, during skeletal muscle regeneration, which is associated with routine maintenance, hypertrophy, and repair, satellite stem cells are activated from their quiescent state, proliferate, and finally differentiate (Adams, 2006; Zammit *et al.*, 2006). Because VEGF is a mitogen for ECs (Leung *et al.*, 1989), we tested whether it might also regulate myoblast proliferation. Using CFDASE cell labeling as a measure of cell proliferation, we observed that sFlt1-treated cells retained significantly more CFDASE than control cells, indicating that VEGF neutralization reduced the division rate of C2C12 cells (Figure 7D). We found no difference in CFDASE retention between control and VEGF-treated cells (data not shown); however, this was not surprising given that the cells were grown in 10% FBS and were likely replicating at maximum capacity. Given the tendency of C2C12 cells to undergo differentiation and thereby enter G0 at lower serum concentrations, we did not test the mitogenic effects of VEGF at low serum concentrations.

DISCUSSION

The formation of vascular systems is essential for normal growth, differentiation, and organ function where the vascular bed is specialized to meet the needs of the organ it supplies. To achieve the high degree of structural organization necessary to serve the specific tissue requirements, blood vessel formation is tightly regulated by the specific expression and secretion of factors such as VEGF. VEGF has been shown to be expressed at varying levels in a cell-specific manner in virtually all vascularized adult tissues (Maharaj *et al.*, 2006) and is up-regulated in a number of cell types in response to hypoxic conditions (Liu *et al.*, 1995; Forsythe *et al.*, 1996; Mazure *et al.*, 1996). The data presented in this report demonstrate a novel regulatory mechanism by which VEGF is up-regulated during skeletal muscle differentiation by the myogenic regulatory factor, MyoD. Moreover, although VEGF is well established as a modulator of blood vessel formation (Ferrara *et al.*, 2003), the expression of VEGF receptors by many cell types in addition to EC points to nonvascular roles for VEGF in the adult (Maharaj *et al.*, 2006; D'Amore, 2007). Indeed, our data suggest that VEGF mediates skeletal myogenesis and that VEGF expressed by differentiating myogenic precursors functions in an autocrine manner.

Through differential mRNA splicing, the single murine VEGF gene gives rise to at least three isoforms including, VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈ (Ferrara *et al.*, 1992; Shima *et al.*, 1996). VEGF₁₂₀ lacks a heparan sulfate binding site and is therefore freely diffusible, whereas VEGF₁₈₈, with two heparan sulfate binding sites, associates with the cell surface and extracellular matrix, and VEGF₁₆₄ has intermediate properties (Park *et al.*, 1993; Ferrara and Davis-Smyth, 1997). The various VEGF isoforms play distinct roles in vascular development, and their patterns of expression vary during organ development and in the adult (Ng *et al.*, 2001; Robinson and Stringer, 2001; Skryabin *et al.*, 2004). Our data demonstrating VEGF₁₈₈ and VEGF₁₆₄ expression in skeletal muscle is consistent with a number of other studies in human, rat, and mouse (Ng *et al.*, 2001; Ishii *et al.*, 2002; Jensen *et al.*, 2004; Maharaj *et al.*, 2006). Given that skeletal muscle is subjected to a considerable amount of daily stresses including exercise-induced hypoxia (Hoppeler, 1999) and structural damage (Russell *et al.*, 1992), necessitating angiogenesis, VEGF expression in this tissue is not surprising. In addition, we demonstrate that multipotent cell lines (C2C12 and 10T1/2), which under the appropriate conditions are molecularly similar to myogenic satellite stem cells, express all three VEGF isoforms. These data suggest that myogenic precursors, in addition to expressing the sequestered forms of VEGF (VEGF₁₈₈ and VEGF₁₆₄), may express diffusible VEGF₁₂₀, which could function to attract distant vessels to sites of myoblast differentiation. Although multiple reports demonstrate variable tissue-specific isoform expression patterns for VEGF, the mechanism controlling this process remains unknown.

HIF1 serves as a master regulator for the expression of a number of genes involved in the hypoxic response, including glucose transporters and glycolytic enzymes as well as VEGF (Damert *et al.*, 1997; Minchenko *et al.*, 2002; Mobasher *et al.*, 2005), and hypoxic regulation of VEGF expression via HIF-1 is the best characterized mechanism of VEGF control. Interestingly, deletion of the hypoxia-response element in the VEGF promoter in mice results in a viable mouse with only minor phenotypes (Oosthuysen *et al.*, 2001). In contrast, VEGF^{-/-} and ^{+/-} mice are embryonic lethal (Ferrara *et al.*, 1996). Thus, the dispensable nature of HIF-1 suggests

that HIF-1 is much less important in the regulation of VEGF than previously suspected. Furthermore, there are a small but growing number of reports documenting mechanisms that modulate VEGF expression in normoxic conditions. For instance, myogenic Akt signaling controls both muscle fiber hypertrophy and VEGF synthesis, independent of HIF-1 activity, illustrating a mechanism through which blood vessel recruitment can be coupled to normal tissue growth (Takahashi *et al.*, 2002). Moreover, analysis of VEGF mRNA levels across an array of adult tissues demonstrates a wide variability in the expression pattern for VEGF (Ng *et al.*, 2001), suggesting complex transcriptional modulation of this gene. TESS promoter analysis (<http://www.cbil.upenn.edu/cgi-bin/teess/teess>) of the human VEGF promoter reveals a wide array of cell-type specific putative transcriptional regulatory sites for transcription factors, such as c-Myb (involved in proliferation and differentiation of hematopoietic progenitor cells), myogenin (involved in differentiation of skeletal muscle), T-cell factor (TCF; involved in T-cell specific differentiation), and peroxisome proliferator-activated receptor (PPAR; transcription factor specific for liver, kidney, heart, and brown adipose tissue), to name only a few.

Our observation of a number of putative CANNTG MyoD-binding sites within 9 kb upstream of the VEGF transcriptional start site motivated us to investigate if skeletal muscle-specific regulation of VEGF exists independent of hypoxic regulation. In skeletal muscle, MyoD functions in an instructive chromatin context and directly regulates genes expressed throughout the myogenic program (McKinsey *et al.*, 2001; Pownall *et al.*, 2002). For example, expression of MyoD is sufficient to convert fibroblast and adipoblast cells into skeletal muscle cells (Tapscott *et al.*, 1988). Indeed, our analysis revealed that VEGF is transcriptionally up-regulated during skeletal muscle differentiation via the myogenic transcription factor MyoD and its binding partners, E12 and E47. Many studies have demonstrated VEGF up-regulation in skeletal muscle regeneration, transplantation, and exercise models (Rissanen *et al.*, 2002; Smythe *et al.*, 2002; Arsic *et al.*, 2004; Prior *et al.*, 2004; Yan *et al.*, 2005; Wagatsuma, 2007; Xia *et al.*, 2006), and our report expands on that knowledge by providing a specific mechanism by which VEGF is up-regulated during normal skeletal muscle differentiation. Indeed, in light of the many putative tissue-specific transcription factor binding sites located in the VEGF promoter, we speculate that the differentiation-dependent expression of VEGF that we have documented in skeletal muscle may be a paradigm for VEGF regulation in adult tissues.

The most studied aspects of VEGF in skeletal muscle involves its role in inducing angiogenesis during muscle formation and after bouts of exercise (Wagner, 2001; Haas, 2002; Bloor, 2005). Two contradicting studies have previously attempted to examine the effects of VEGF on skeletal myogenesis. One study suggests that VEGF strongly affects myocyte migration and survival but reports no effect on myogenic marker expression (Germani *et al.*, 2003). The interpretation of these results remains complicated because the authors report very early expression of MHC, in contrast to a large number of reports that demonstrate that MHC is only detectable by Western blotting in C2C12 cells at 2 to 3 d of differentiation (Miller, 1990; Andres and Walsh, 1996). Another report demonstrated increased proliferation and decreased apoptosis in C2C12 cells in response to exogenous VEGF, as well as an increase in myotube number, size, and multinucleation (Arsic *et al.*, 2004); however, this group did not examine a possible autocrine role for VEGF.

We have comprehensively examined the role and regulation of VEGF during skeletal myogenesis and demonstrate that VEGF stimulation increases myotube number, myogenic marker expression, and myotube size, whereas inhibition of VEGF signaling decreases these processes. These data indicate that VEGF may be essential for proper myogenic differentiation. Given the ubiquitous distribution of VEGF, we speculate that VEGF may perform similar autocrine roles for a number of developmental processes. For instance, VEGF is overexpressed in chondrogenic and osteogenic differentiation cascades, and inhibition of VEGF activity results in reduced bone differentiation, suggesting that this factor plays an important role during cartilage and bone formation (Mayer *et al.*, 2005; Bluteau *et al.*, 2007). Moreover, differentiating kidney podocytes strongly up-regulate VEGF and VEGFR2, and treatment with VEGF reduces podocyte apoptosis ~40%, suggesting VEGF promotes survival in these cells through VEGFR2 (Guan *et al.*, 2006). Genetic overexpression of VEGF in adult rats results in an approximately twofold increase in hippocampal neurogenesis associated with improved cognition, whereas inhibition of VEGF expression by RNA interference completely blocks the environmental induction of neurogenesis, suggesting that VEGF serves as a mediator of neurogenesis and cognition (During and Cao, 2006). It seems clear that future studies will elucidate more instances where VEGF is utilized for the function of nonvascular cell types.

The coordinated regulation of the VEGF receptors during skeletal myogenesis possibly reflects the underlying biology of the system. Our data suggest that proliferating, undifferentiated C2C12 cells predominantly express VEGFR1; however, differentiating C2C12 cells down-regulate VEGFR1 and dramatically increase the expression of VEGFR2. Because the differentiated C2C12 cells have completely exited the cell cycle, VEGFR2 signaling no longer leads to cell proliferation but instead appears to contribute to myogenic differentiation. A similar induction of VEGFR2 expression has been observed in differentiation of podocytes (Guan *et al.*, 2006). The expression of VEGFR2 has been shown in other systems to be induced by VEGF (Enholm *et al.*, 2001), and given our data indicate that VEGF is up-regulated during myogenic differentiation, this may account for the observed increased VEGFR2 expression. Our findings that VEGF can mediate myogenic differentiation and survival are consistent with an autocrine role for VEGFR2 signaling in differentiating and differentiated skeletal muscle. At the same time, the VEGF produced by the differentiating myocytes is necessary for the vascularization of the developing skeletal muscle.

Although these results are important in elucidating the molecular mechanisms controlling tissue-specific vascular development and maintenance, they also present broader medical implications for anti-angiogenic treatment for diseases such as cancer and macular degeneration. Patients undergoing long-term anti-VEGF therapy may experience unexpected side effects due to interference with normal vessel regrowth/stability in skeletal muscle tissue and/or prolonged muscle regeneration time after injury. For example, although developmental differentiation of skeletal muscle does not occur in the adult, day-to-day wear and tear of adult skeletal muscle accounts for ~1–2% replacement of muscle fibers per week (Schmalbruch and Lewis, 2000)—processes that involve satellite stem cell activation and subsequent myogenic differentiation and that could be susceptible to anti-VEGF therapies. Consistent with this speculation, patients treated with Avastin report asthenia, which, though nonspecific, may result from reduced muscle regeneration. Because of the low rate of skeletal muscle turnover/

repair, abnormalities might be expected to require extended treatment to develop. In addition, because patients receiving Avastin often exhibit a spectrum of medical issues, subtle changes in muscle function might be difficult to detect. A beneficial effect of VEGF overexpression has been reported for skeletal muscle regeneration through both proangiogenic effects and myogenic regenerative effects (Arsic *et al.*, 2004; Messina *et al.*, 2007). These observations suggest that anti-VEGF drugs could inhibit muscle regeneration through dual mechanisms—blocking angiogenesis and blocking satellite stem cell activation and subsequent myogenesis.

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