SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Cell cultures

Mouse skeletal muscle endothelial cells (SMECs){Ieronimakis, 2008 #457} were cultured in DMEM with 4.5 g/L glucose and ultraglutamine 1 (Lonza), containing 10% fetal bovine serum (FBS) (Sigma), 10 ng/ml mouse recombinant $VEGF_{164}$ (VEGF-A, R&D system), 1 mmol/L sodium pyruvate (Lonza), 10 ml/L penicillin-streptomycin (Lonza). Cells were used between passages 7 and 14.

Human umbilical vein ECs (HUVECs) were purchased from Lonza. Cells were cultured in EGM-2 (EBM-2 plus with EGM-2 SingleQuots of growth supplements, Lonza) containing 2% FBS (complete medium) and used between passages 3 and 6. HUVECs were transfected, using jetPEI^{TM}-HUVEC (Polyplus transfection), with the pcDNA3.1 vector containing rat full length *TrkC* receptor{Esteban, 2006 #459} and encoding ampicillin resistance. This vector was kindly donated by Prof. Lino Tessarollo (National Cancer Institute, NIH, Frederick, Maryland, USA).

Human vascular smooth muscle cells (hVSMCs) were a kind gift of of Dr. Yanhua Hu (King's College, London, UK). Cells were cultured in DMEM with ultraglutamine 1 containing 10% FBS and 10 ml/L penicillin-streptomycin, as previously described{Hu, 1999 #460;Xu, 1997 #461}.

In all *in vitro* experiments, culture media were supplemented with either human recombinant NT-3 (50 and/or 100 ng/ml, Promega) or vehicle (PBS). In some *in vitro* experiments 50 µmol/L LY294002 (Calbiochem) or vehicle (DMSO, Sigma), and 6 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME, Sigma) or N^G-nitro-D-arginine methyl ester (D-NAME, Sigma) were added to culture media 30 minutes before NT-3 or its vehicle.

In vitro functional assays

Cell proliferation. SMECs ($2x10^3$ cells per well) were seeded in 96-well plates until adherent. Then, cells were incubated in their own medium without mouse recombinant $VEGF_{164}$, containing either human recombinant NT-3 (50 and 100 ng/ml) or vehicle (PBS) in presence of 5-bromodeoxyuridine (BrdU) for 24 hours at 37℃, 5% CO_2. *TrkC*-transfected HUVECs ($5x10^3$ cells per well) were

seeded in 96-well plates. Next, adherent HUVECs were incubated (at 37℃, 5% CO_2) in EGM2 0% FBS, containing either human recombinant NT-3 (50 and 100 ng/ml) or vehicle (PBS) in presence of BrdU for 24 hours. BrdU incorporation was detected using the Cell Proliferation ELISA, BrdU colorimetric kit (Roche), according to the manufacturer's instruction. Five replicates of each condition were performed. Experiment was repeated three times.

Apoptosis assay. SMECs ($7x10^3$ per well) were seeded on 0.5% gelatine-coated cover slips in 24 well-plate until adherent. Next, cells were incubated in their medium with 0% FBS and without mouse recombinant $VEGF_{164}$ in presence of either NT-3 (50 and 100 ng/ml) or vehicle (PBS) at 37℃, 5% CO_2. After 24 hours, cells were fixed with 4% paraformaldehyde and TUNEL assay (Roche) was performed according to the manufacturer's instructions. Data are expressed as the average percentage of TUNEL-positive nuclei per total nuclei. The experiment was performed in triplicate for each condition and repeated 3 times.

Cell migration. The ability of NT-3 to stimulate the migration of SMECs and *TrkC*-transfected HUVECs was evaluated by scratch assay{Barcelos, 2009 #32}. Cells were seeded on 48-well plates. When cells reached confluence, a central scratch was generated by scraping cells away with a p1000 pipette tip. Cells were gently washed with DPBS to remove debris. Next, SMECs were incubated in DMEM with10% FBS, without mouse recombinant $VEGF_{164}$ and with 2mM hydroxyurea (Sigma), to induce growth arrest. HUVECs were incubated in EGM2 with 0% FBS and 2mM hydroxyurea. NT-3 (50 and 100 ng/ml) or vehicle (PBS) was added to SMECs and HUVECs. Plates were incubated at 37℃ and 5% CO_2 and photographed after 24 hours. Gap closure was measured under an inverted phase-contrast microscope. The distance between migrating fronts was measured using the Image-Pro Plus software (Media Cybernetics), as previously described{Barcelos, 2009 #32}. Each condition was run in quadruplicate and the assay was repeated 3 times.

Furthermore, the capacity of human vascular smooth muscle cells (hVSMCs) to migrate toward a gradient of NT-3 was evaluated by using fibronectin-coated (10µg/ml, Sigma) 24-well-plate transwell migration inserts (Corning, Artington, UK) with a polycarbonate membrane of 8-µm pore size. hVSMCs were trypsinized and resuspended in DMEM containing 0.1% bovine serum albumin

(BSA, Sigma). Cells ($1.5x10^4$ per well) were placed in the upper chamber, while DMEM containing 0.1% BSA and either human recombinant NT-3 (50 and 100 ng/ml) or vehicle (PBS) were placed in the lower chamber. Cells were incubated in a humidified incubator at 37℃ and 5% CO_2 for 16 hours. Non-migrated cells were removed by gently wiping the upper surface of the membrane with a cotton swab. Membranes were fixed in 5% methanol, excised with a scalpel and mounted on microscope slides with mounting medium containing Dapi (Vectashield, Vector Technologies). Percentage of migration was assessed by counting the number of cells present on the lower surface of the membrane in 8 random view fields (magnification 100X). Each condition was performed in triplicate and the assay was repeated 3 times.

Endothelial network formation on Matrigel. SMECs or *TrkC*-transfected HUVECs were resuspended in their own cell media containing either human recombinant NT-3 (50 and 100 ng/ml) or vehicle (PBS) and next seeded ($4×10^4$ cells per well) in 48-well plates pre-coated with 150µl of growth factor reduced Matrigel™ (BD Biosciences) thick gel,. In inhibition experiments, 50 µmol/L LY294002 (Calbiochem) or vehicle (DMSO, Sigma), and 6 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME, Sigma) or N^G-nitro-D-arginine methyl ester (D-NAME, Sigma) were added to cell media 30 minutes before NT-3 or its vehicle. Plates were incubated at 37℃ and 5% CO_2 and photographed at different time points from seeding. Endothelial network formation was examined under an inverted phase-contrast microscope. Network formation was quantified by counting the number of branches per view field using Image Pro-Plus software (Media Cybernetics). Each condition was performed in triplicate and the assay repeated 3 times.

TrkC receptor expression

SMECs ($5X10^3$ cells per well), *TrkC*-transfected HUVECs ($7X10^3$ cells per well) or hVSMCs ($2.5X10^3$ cells per well) were seeded on 0.5% gelatine-coated cover slips in 24 well-plate. Next, adherent cells were fixed with 2% paraformaldehyde for 30 minutes at room temperature. After 30 minutes of incubation with 10% goat serum in PBS, cells were overnight incubated with a rabbit polyclonal antibody targeted to rat and mouse TrkC (AbCam) at 4℃, followed by goat anti-rabbit Alexa Fluor 568 (Invitrogen).Nuclei were visualized by Dapi (4',6-diamidino-2-phenylindole, Sigma-

Aldrich) staining. Slides were mounted with Fluoromount-G mounting medium (SouthernBiotech). Images were captured at 200X magnification using an Olympus BX40 fluorescence microscope.

TrkC receptor expression was also evaluated in mouse normoperfused adductor muscles by immunohistochemistry. Paraffin-embedded sections (3μm thick) were deparaffinised. Antigen retrieval was performed on de-waxed sections by microwave heating in 10mM citrate buffer pH 6 for 12 minutes. After inhibition of endogenous peroxidases by incubating tissue sections with 3% H_2O_2/H_2O for 10 minutes, unspecific staining was blocked with 10% swine serum in PBS for 30 minutes. Specimens were subsequently incubated with a goat anti-mouse TrkC primary antibody (1:100; R&D System) overnight at 4℃. Specific prim ary antibody binding was revealed using LSAB2 System, Peroxidase Kit (DakoCytomation). Staining was developed by using DAB chromogen (DakoCytomation). Cell nuclei were counterstained with Mayer's haematoxylin.

Adenoviruses

An adenovirus carrying the coding sequence of *rat Neurotrophin-3* (*Ad.NT-3*) was kindly provided by Dr. H. David Shine (Baylor College of Medicine, Houston, Texas, USA). Replication-deficient adenoviruses were generated by site-specific FLP-mediated recombination of the co-transfected shuttle and genomic plasmids in 293 cells. Viral stocks were amplified, CsCl purified, and titrated as previously described{Caporali, 2008 #320;Caporali, 2007 #334}. Empty vectors called *Ad.Null* or *eGFP* expressing vector (*Ad.eGFP*) were used as negative controls as indicated. An Akt adenovirus expressing a dominant-negative mutant for the phosphorylation sites Threonine 308 and Serine 473, Akt T308A/S473A (*Ad.DNAkt*){Condorelli, 2002 #19;Emanueli, 2004 #20;Condorelli, 2002 #19}, was used. The capacity of *Ad.NT-3* to infect *in vivo* was evaluated in mouse ischemic adductor muscle (*vide infra*) by RT-PCR using primers that detect rat and not murine *NT-3*. PCR products were run on 1.2% agarose gels to confirm transgene expression. All PCR primers are reported below in this section.

Animal procedures

All the experiments involving mice and rats were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and with prior approval of the UK Home Office and the University of Bristol.

Male Wistar rats (Harlan, UK) were used at 300-350g. Male CD1 mice (Charles River Laboratories, Morgate, UK) were used at 25-35g. Animals had unrestricted access to standard chow and drinking water. All animals were housed at constant room temperature (24 ± 1℃) with a 12h light/12h dark cycle.

Rat Mesenteric Angiogenesis Assay

Surgery was performed as previously described{Wang, 2004 #21;Stone, 2009 #22;Benest, 2008 #23}. A mesenteric panel was exposed under an intravital microscope (Leica DMIL). The panel was imaged and either *Ad.NT-3* or *Ad.eGFP* was injected into the surrounding fat pad as 25 μl injections at a concentration of 1×10^8 p.f.u. We have previously shown that this results in transfection of adipocytes{Wang, 2004 #21}. Five μl of Monastral blue (0.6%, diluted in saline) was injected into the fat pad on either side of the virus-injected panel. The intestine was replaced and the animal sutured and allowed to recover. Six days later, the animal was re-anaesthetised, the mesentery exposed and the virus-injected panel located and imaged as above. The panel was fixed *in vivo* with 4% paraformaldehyde for 5 minutes, and the rat was sacrificed by cervical dislocation. The individual mesenteric panel was then excised and fixed with 4% paraformaldehyde for 30 minutes.

Immunofluorescent staining of mesenteric panels

After fixation, the mesenteric panel was washed 6 times with 0.5% Triton-X100 in phosphate buffered Saline (0.5% PBX) and blocked at room temperature with 1% BSA (Sigma) in 0.5% PBX for 1h. The mesentery was incubated with biotinylated isolectin B4 (10μg/ml; Griffonia Simplificolia; Molecular Probes), mouse monoclonal antibodies to NG2 (1:200; Chemicon) and rabbit polyclonal antibodies to α-smooth muscle actin (1:125; AbCam). All primary antibodies were diluted in blocking solution and incubated overnight at 4℃ on a rocker. The mesentery was washed in 0.5% PBX for 6 x 10 minutes and incubated in block solution containing TRITC-streptavidin (1:1000; Molecular Probes), goat-anti-rabbit Alexa-350 (1:500; Molecular Probes) and goat-anti-mouse Alexa-488 (1:500; Molecular Probes), for 2h at room temperature. After washing for 6 x 10 minutes in 0.5% PBX, the mesentery was flat mounted using VectaShield (VectorLab, Peterborough, UK).

Flat mounted mesenteries were scanned with a Leica Confocal Microscope (Leica Confocal TCS-NT system, Leica, Bucks, UK).

Microvessel analyses

Perfused vessels were imaged by intravital microscopy and analysis was carried out offline using Openlab 3.1 Software (Improvision, Coventry, UK). The percentage of the functional vessel area (%FVA) was measuared. Increase in percentage vessel area (% angiogenic index) was calculated from the functional vessel area (FVA) on day 6 and 0 by using this formula:

$$\% \text{ Area Increase} = \frac{\text{FVA on day 6} - \text{FVA on day 0}}{\text{FVA on day 0}} \times 100$$

For each mesentery, five view fields were randomly selected (at 630× magnification) and Openlab software was used to analyze vessel parameters between two adjacent branch points from confocal stack images. Total vessels were counted and labelled and the diameter and length of each vessel was measured. Pericyte and vascular smooth muscle cell (VSMC) coverage was calculated as percentage of the vessel area. A minimum of n=5 rats per each group were used for these analyses.

Mouse hindlimb ischemia model

Left limb ischemia was induced in anaesthetized CD1 male mice, as described{Salis, 2004 #27;Invernici, 2007 #26;Gadau, 2006 #25;Emanueli, 2007 #24}. After animals were randomly allocated into treatment groups, ischemia of the left hind limb was induced by ligature and electro-coagulation of the left common femoral artery proximal to the bifurcation of the superficial and deep femoral artery. Immediately after this, the left adductor received three injections of either *Ad.Null* or *Ad.NT-3* corresponding to 1×10^8 p.f.u. (in a total of 21 μl) per animal.

Blood flow (BF) measurements

Superficial BF of the ischemic and contralateral foot was analyzed at different time-points (day 0, 7 and 14) from ischemia induction by colour laser Doppler (Lisca Perimed, Sweden) and the ratio between BF in the ischemic foot and BF in the contralateral foot was calculated and used as an

index of % BF recovery{Emanueli, 2001 #367;Emanueli, 2002 #257;Salis, 2004 #321}. A minimum of n=12 mice per group were studied unless differently stated.

Histology and immunohistochemistry

At day 14 after induction of ischemia, mice were anaesthetized (2,2,2-tribromoethanol, Sigma) and the descending thoracic aorta was cannulated for perfusion/fixation of the hind-limbs. Perfusion was performed with 10 ml of PBS/heparin and then 20 ml of 10% formalin. Adductor muscles were harvested from the ischemic and contralateral sides and kept in 4% formalin for 24 hours. Then, muscles were embedded in paraffin.

Capillary density was examined on 4μm-thick transverse paraffin-embedded sections following staining with Hematoxylin and Eosin (H&E) and observed at 1000× magnification. Capillary density was counted using an ocular graticle, as previously described{Emanueli, 2001 #367;Emanueli, 2002 #257;Salis, 2004 #321}. The number of capillaries was also measured in fluorescent sections stained with isolectin B4 (Griffonia Simplificolia; Molecular Probes) to identify ECs and with Dapi (Sigma Aldrich) to identify nuclei. A minimum of n=5 mice per each group was used for these analyses.

Arteriole density was evaluated using a mouse monoclonal anti-α-smooth muscle actin antibody (DakoCytomation) and isolectin B4 (for ECs). Analyses were performed at 400× magnification. The number of arterioles was expressed as the ratio between ischemic and contralateral muscle per mm^2. A minimum of n=5 mice per group were used for these analyses.

Proliferating ECs were counted in ischemic adductor muscles injected with either *Ad.NT-3* or *Ad.Null*. Sections were stained with a mouse monoclonal anti-PCNA antibody (Proliferating Cell Nuclear Antigen, clone PC10, Dako) and biotin-conjugated isolectin B4 followed by goat anti-mouse Alexa Fluor 488 and Stp-Alexa Fluor 568. Nuclei were visualized by Dapi. A minimum of n=5 mice per group were used for these analyses.

***In vivo* Akt inhibition**

To functionally inhibit the action of Akt in *Ad.NT-3*-induced neovascularization, an *in vivo* cotransfection experiment with *Ad.dominant negative-AktT308A/S473A* (*Ad.DNAkt*) was performed, as previously described{Condorelli, 2002 #19}{Emanueli, 2004 #20}. Adductors

received 10^8 p.f.u. of *Ad.NT-3* or *Ad.Null* (as control for NT-3) in combination with either *Ad.DNAkt* or *Ad.Null* (as control for *Ad.DNAkt*) (each at 10^8 p.f.u.). Neovascularization was evaluated after 2 weeks in 6 mice per group.

***In vivo* NOS inhibition**

Capillary and arteriole densities were counted after 2 weeks from *Ad.NT-3* or *Ad.Null* gene transfer (each at 10^8 p.f.u.) in combination with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 1.4 mmol/kg body weight, IP, daily in drinking water) or the inactive enantiomer D-NAME{Emanueli, 2004 #20}. A minimum of n=6 mice per group was used for these analyses{Emanueli, 2004 #20}.

***In vivo* NT-3 neutralization**

In order to assess the role of endogenous NT-3 in the spontaneous neovascularization response to ischemia, a soluble TrkC receptor domain which neutralizes NT-3 (TrkCd5) was used. Recombinant TrkCd5 consisting of residues 241-367 with an N-terminal histidine tag was prepared using the vector pET15b in Bl2 (2E3) cells. The protein was refolded and purified as per TrkAd5{Robertson, 2001 #29} and TrkBd5{Banfield, 2001 #462}. The purified protein was at a concentration of 1.05 mg/ml in 20 mM sodium phosphate, 100mM NaCl, 10% glycerol (pH 7.6). TrkCd5 or vehicle was injected in ischemic mice (2 mg/kg body weight, IP, every 4 days starting on the day of the ischemia induction). Capillary and arteriole densities and blood flow recovery were analysed 2 weeks after surgery. A minimum of n=6 mice per group were used.

RNA extraction and RT-PCR

Total RNA was isolated from murine muscles using Tri Reagent (Sigma) and DNase (Qiagen) treated according to the manufacturer's instructions. RNA quality was confirmed using the RNA Nano LabChip in a bioanalyzer (Agilent). Five hundred nanograms of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). One microliter of each cDNA preparation was amplified by PCR using the following set of primers: *18S rRNA* (forward: 5'-TAGAGGGACAAGTGGCGTTC-3', reverse: 5'-TGTACAAAGGGCAGGGACTT-3', amplified PCR products: 200 bp); *mouse TrkC* (forward: 5'-CCTGACACAGTGGTCATTGG-3', reverse: 5'-TATGCTCATGCTGCAGGTTC-3', amplified PCR products: 307 bp); *rat TrkC (*forward: 5'-

TGCCTGATGTGGACTGGATA-3', reverse: 5'- CAGCCCTCTGAGACTTCACC-3', amplified PCR products: 373 bp); *rat NT-3* (forward: 5'-GATCCAGGCGGATATCTTGA-3', reverse: 5'-AATCATCGGCTGGAATTCTG-3', amplified PCR products: 145 bp).

Real time PCR

Quantitative PCR was performed in a LightCycler (Roche, Burgess Hill, UK), using IQ SYBR Green supermix (Qiagen) and the following primers pairs: *18s rRNA* (forward: 5'-TAGAGGGACAAGTGGCGTTC -3', reverse: 5'-TGTACAAAGGGCAGGGACTT-3'); *murine VEGF-A* (forward: 5'-GGAGATCCTTCGAGGAGCACTT-3', reverse: 5'-GGCGATTTAGCAGCAGATATAAGAA-3'); *murine NT-3* (forward: 5'-GGAGTTTGCCGGAAGACTCTC-3', reverse: 5'-GGGTGCTCTGGTAATTTTCCTTA-3'); *murine TrkC* (forward: 5'-CTGAGTGCTACAATCTAAGCCC-3', reverse: 5'-CACACCCCATAGAACTTGACAAT-3').; *murine FGF-2* (forward: 5'-GCGACCCACACGTCAAACTA-3', reverse: 5'-TCCCTTGATAGACACAACTCCTC-3'); *murine FGF-2* (forward: 5'-TGCATCAGTGACGGTAAACCA-3', reverse: 5'-TTCTTCAGCCGTGCAACAATC-3'). All experiments were carried out in triplicate and target mRNA levels were normalized to expression of *18s rRNA* in each sample.

Western blot analyses

Proteins were extracted from SMEC lysates and frozen adductor muscles in cold lysis buffer (50mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 25mM NaF, 5mM NaPPi, 1% Triton, 1% NP40, 0.25% Sodium Deoxycholate, 1mM Na_3VO_4, 0.1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, Complete EDTA-free Roche). Protein concentration was determined by the Bradford protein assay (Biorad). Whole protein extracts (80µg for muscle lysates and 20µg for cell lysates) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF, Amersham-Pharmacia) and then probed with the following antibodies: Ser1177-phospho-eNOS (Cell Signaling, 1:1000), eNOS (Santa Cruz Biotechnology, 1:500), Ser473-phospho-Akt (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), phosphotyrosine clone 4G10 (Upstate Biotechnology, 1:1000), trkC (Upstate Biotechnology, 1:1000). FGF-2 (Santa Cruz, 1:1000). α/β tubulin (Cell Signaling 1:1000) was used as loading control. For detection, goat anti rabbit or anti

mouse (Amersham Pharmacia, 1:5000) or donkey anti goat (Santa Cruz, 1:3000) antibodies conjugated to horseradish peroxidase were used. Immunoreactive bands were visualised using ECL reagent (Amersham Pharmacia).

Immunoprecipitation

Tissue protein extract (1mg) was incubated with primary antibody (TrkC, 1µg, Upstate Biotechnology). To precipitate the immune protein/antibody complexes, ProteinG Plus-Agarose beads (Santa Cruz Biothecnology) were added and incubated over night. Afterwards, the antibody-protein-ProteinG complexes were collected by centrifugation. Pellets were washed twice in TNET buffer (50mM Tris-HCl pH 7.5, 140mM NaCl, 1% Triton X-100) and TNE buffer (50mM Tris-HCl pH 7.5, 140mM NaCl) and then resuspended in SDS-loading buffer to be used for electrophoresis and resolved by SDS-PAGE for subsequent detection by western blot.

Sandwich ELISA

ELISA was performed on proteins extracts from murine adductors and blood serum (n=5 each group). Muscles were processed as described for western blot. For serum, peripheral blood (about 1ml) was withdrawn from the beating heart of terminally anesthetized mice. Blood was kept for 15 minutes at 37℃ and a further 15 minutes at 4℃. Su bsequently, blood was centrifuged for 10 minutes at 3000 rpm and the supernatant was collected. Protein concentration was measured by the Bradford protein assay (Biorad). To quantify the concentration of Vascular Endothelial Growth Factor (VEGF), the DuoSet® ELISA Development System (R&D System) was used according to the manufacturer's instructions.

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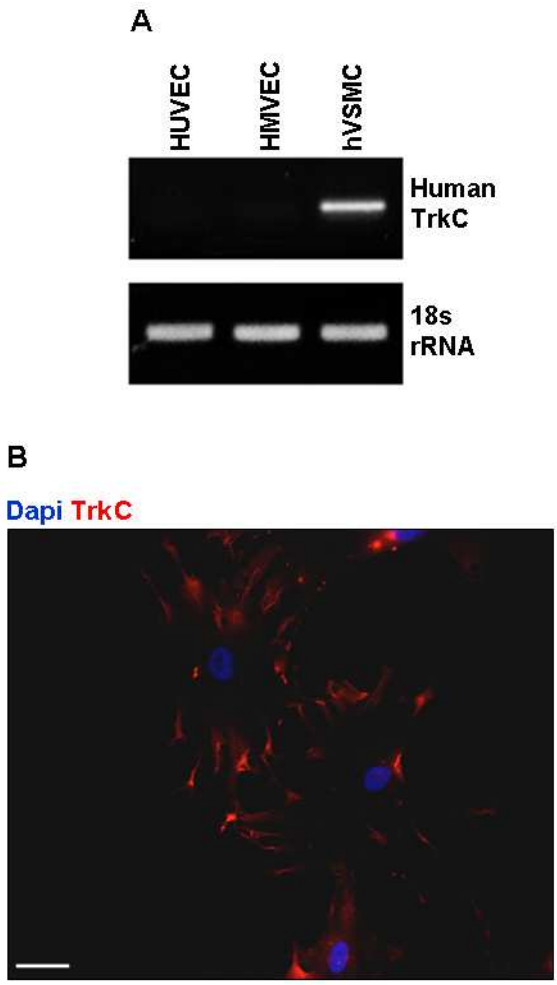
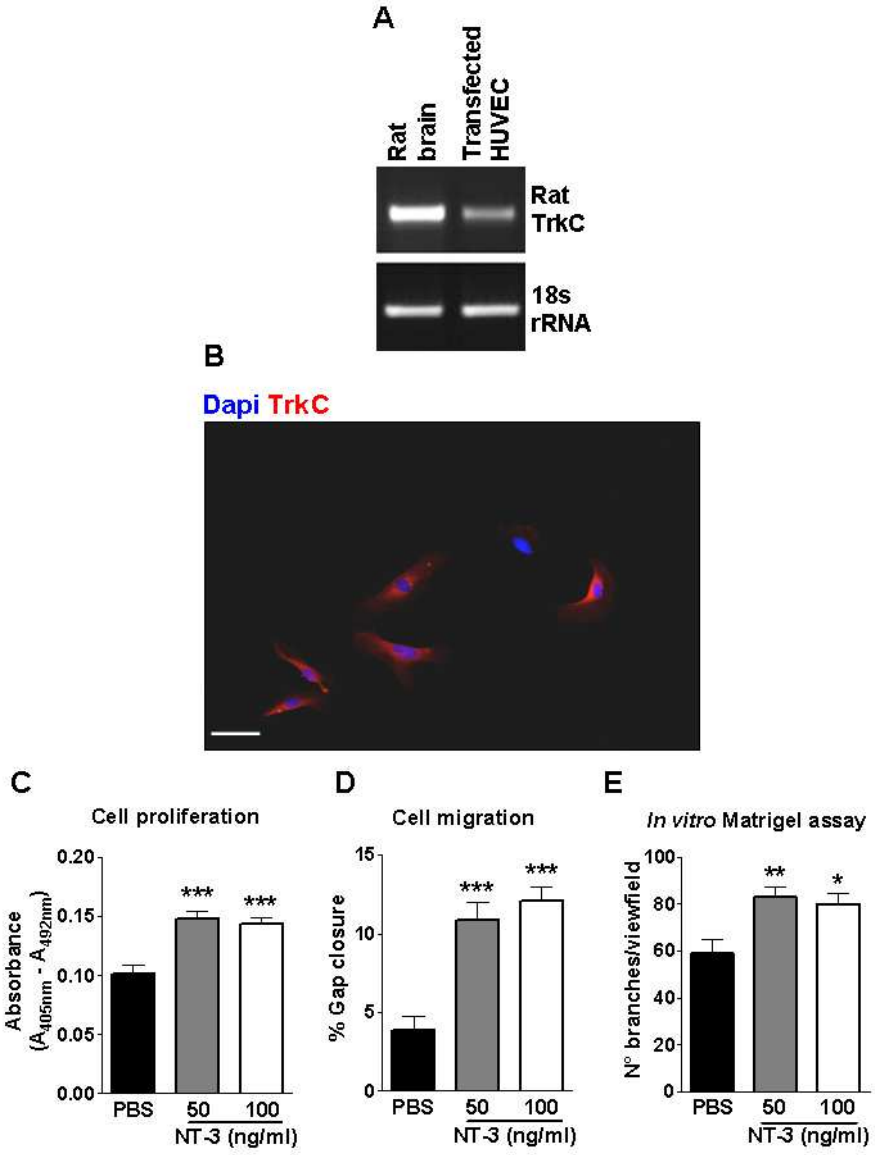
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



Supplemental Figure I

HUVECs and HMVECs do not express TrkC receptor. hVSMCs express TrkC.

(A) RT-PCR image showing *human TrkC* mRNA expression in HUVECs, HMVECs and human vascular smooth muscles cells (hVSMC). *18s ribosomal RNA* (*18s rRNA*) was used as loading control. Bands are representative of three independent experiments. (B) Microphotograph illustrates TrkC receptor expression (stained in red) in hVSMCs. Nuclei are stained with Dapi (blue). Scale bar= 50 µm.



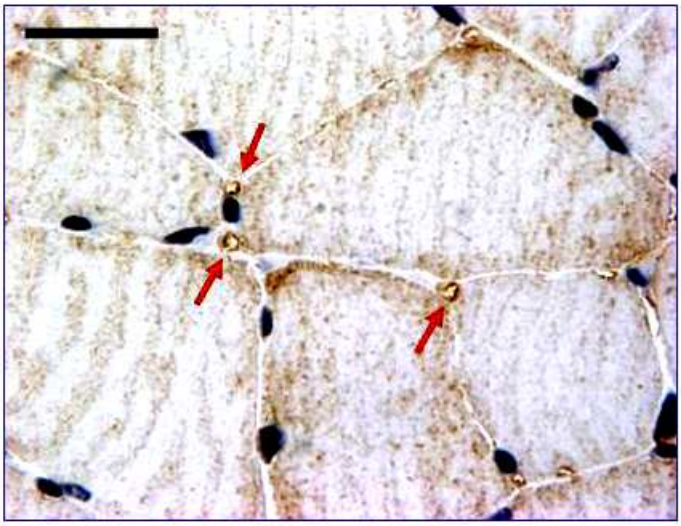
Supplemental Figure II

NT-3 promotes angiogenesis in *TrkC*-transfected HUVECs.

(A) RT-PCR bands presenting *rat TrkC* mRNA expression in rat TrkC-transfected HUVECs. Rat brain was used as positive control. *18s rRNA* was used as loading control. Bands are representative of three independent experiments. (B) TrkC expression was also assessed by immunocytochemistry. Microphotograph shows transfected-HUVEC stained for rat TrkC (red fluorescence). Nuclei were stained with Dapi (blue fluorescence). Scale bar= 50 µm.

(C) Cell proliferation of *TrkC*-transfected HUVECs treated for 24 hours with either vehicle or NT-3 (50-100 ng/ml) was measured by 5-bromodeoxyuridine (BrdU) incorporation assay. (D) Cell migration of *TrkC*-transfected HUVECs was assessed by scratch assay and expressed as

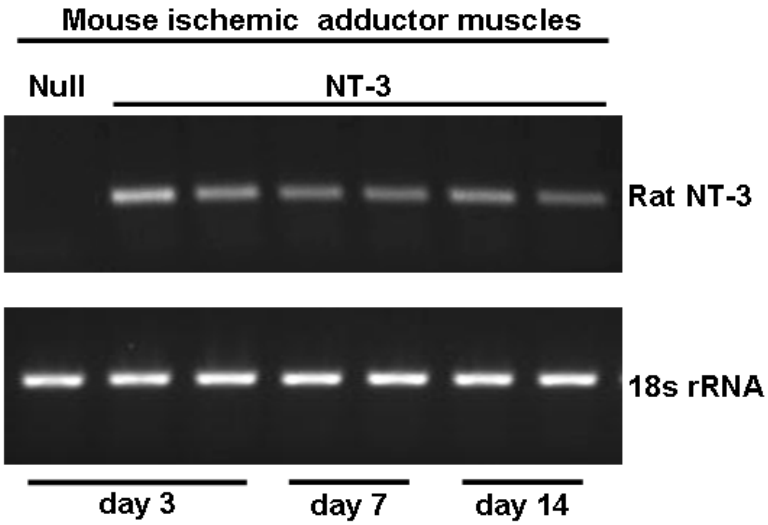
percentage of gap closure. Cells were incubated with either vehicle or NT-3 (50-100 ng/ml) for 24 hours. (E) Endothelial network formation on reduced growth factor Matrigel was assessed following treatment of *TrkC*-transfected HUVECs with NT-3 (50-100 ng/ml) or PBS for 24 hours. The number of branches per view field was counted. All data are mean±SEM, n= 3 for each assay. *P<0.05, **P<0.01 and ***P<0.005 *vs* PBS.



Supplemental Figure III

TrkC receptor is expressed by capillary ECs in skeletal muscles.

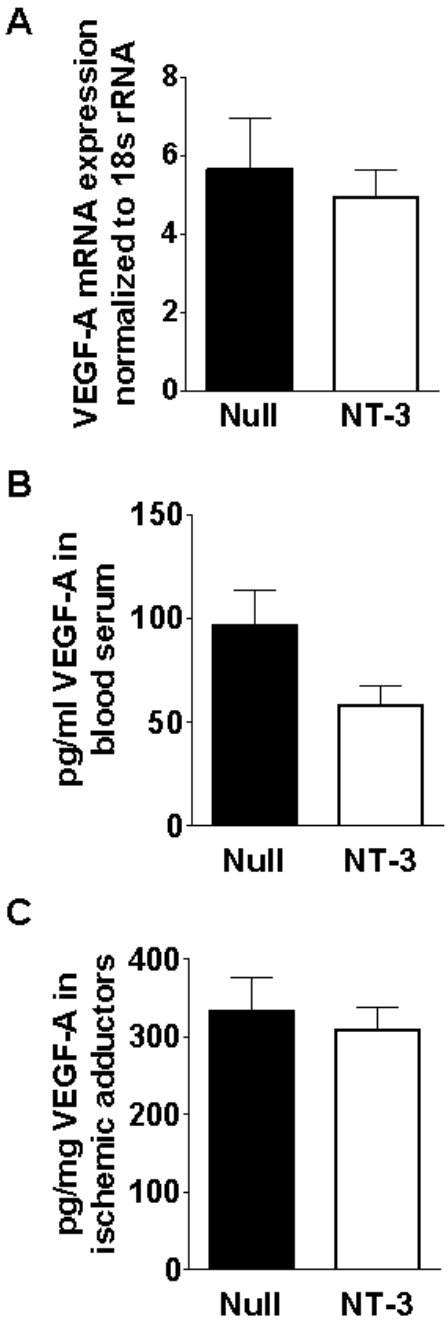
Immunohistochemical staining for the TrkC receptor in normoperfused adductor muscles. Red arrows point to TrkC-positive capillary ECs. Scale bar= 20 µm.



Supplemental Figure IV

***Ad.NT-3* induces expression of transgenic (rat) NT-3 in the mouse ischemic limb muscle.**

RT-PCR images show *rat NT-3* mRNA expression in ischemic adductor muscles at day 3, 7 and 14 after surgery and gene transfer with either *Ad.Null* or *Ad.NT-3*. 18s rRNA was used as loading control. n= 4 mice per group.

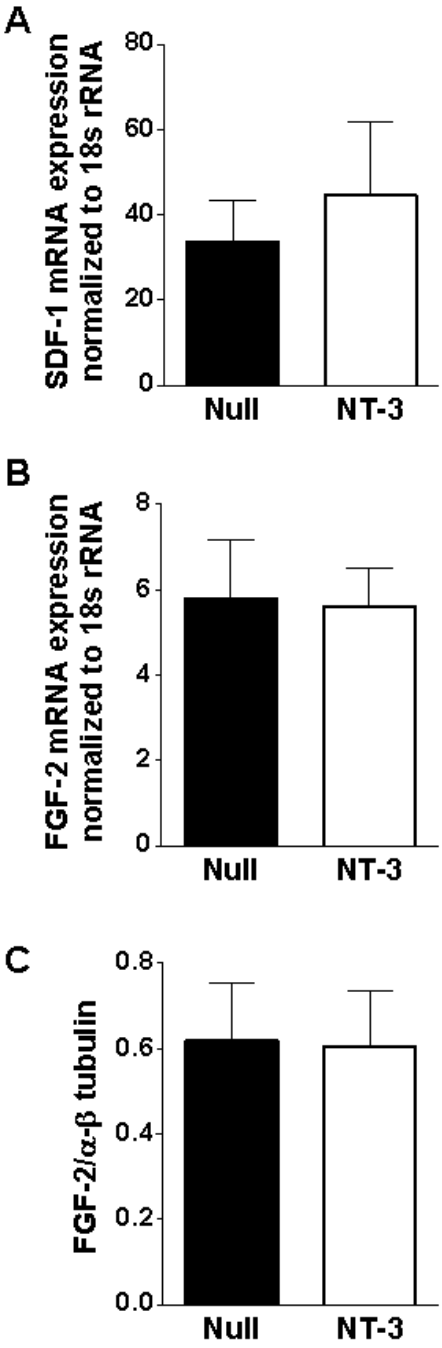


Supplemental Figure V

***NT-3* gene transfer does not affect VEGF-A expression in ischemic adductors.**

Bar graph shows *VEGF-A* gene expression in adductor muscles receiving either *Ad.Null* or *Ad.NT-3* at day 3 post-ischemia. Values are mean±SEM. Real time PCR was performed on n= 5 mice per group (A). Bar graphs display VEGF-A protein expression (measured by ELISA) in blood serum (B)

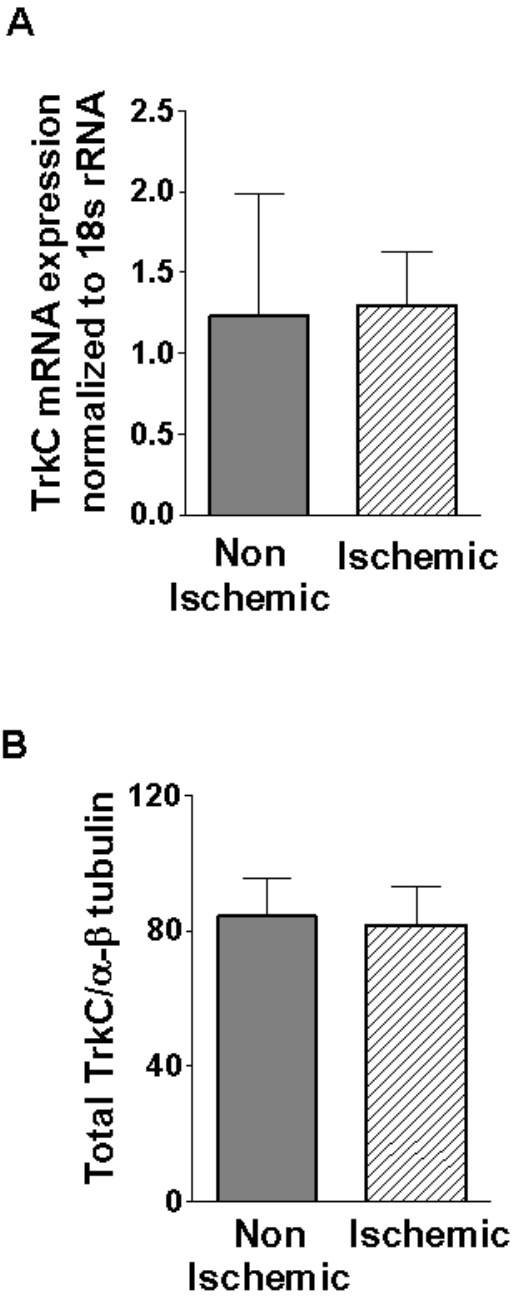
and ischemic adductors injected with *Ad.Null* or *Ad.NT-3* after 3 days from the surgery (C). The assay was performed on n= 5 mice per group. Values are mean±SEM.



Supplemental Figure VI

NT-3 does not change SDF-1 and FGF-2 expression levels *in vivo*.

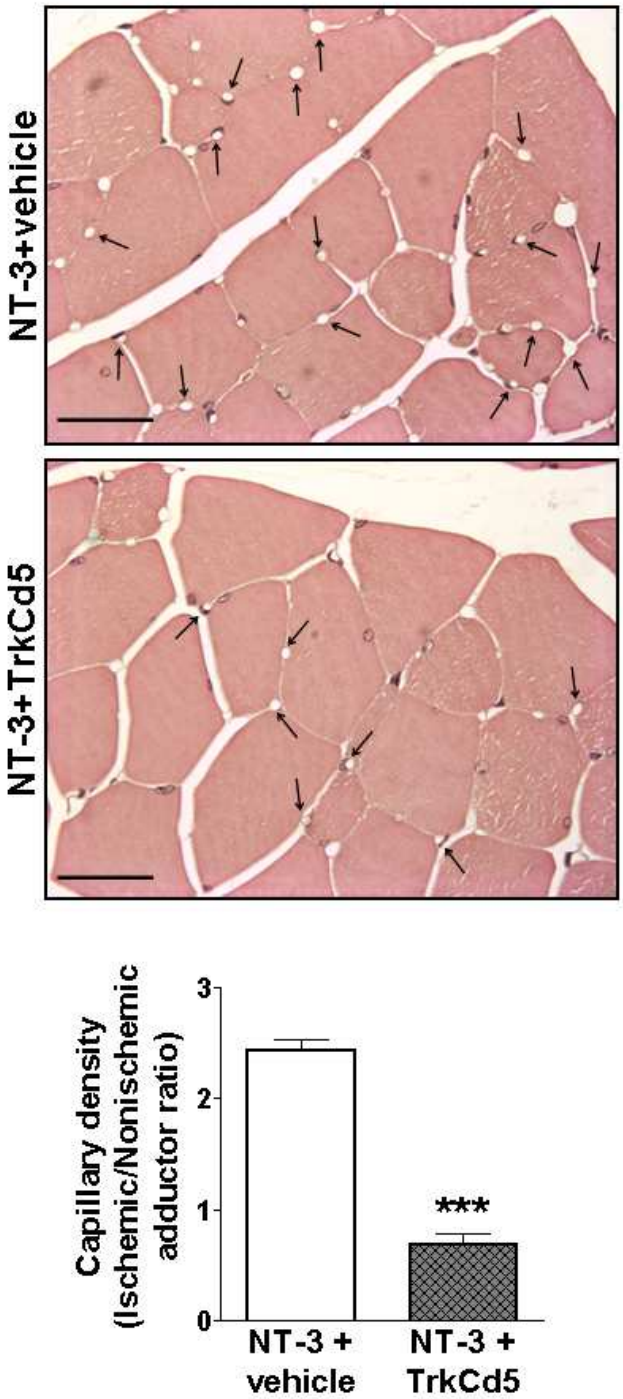
Bar graphs show *SDF-1* (A) and *FGF-2* (B) mRNA expression at day 3 post-ischemia in adductor muscles injected with either *Ad.Null* or *Ad.NT-3*. (C) Bar graph displays FGF-2 protein expression at day 3 post-ischemia in adductor muscles injected with either *Ad.Null* or *Ad.NT-3*. Values are mean±SEM, n=4 for each group.



Supplemental Figure VII

Ischemia does not modulate total TrkC expression in skeletal muscles

Bar graphs show *TrkC* mRNA (A) and TrkC protein (B) levels in mouse ischemic muscles. Real time PCR was carried out to measure *TrkC* mRNA level, n= 4 mice per group. *18s rRNA* gene was used for normalisation. Western blot analysis was performed to detect TrkC protein expression, n=3 mice per group. α-β tubulin was used as loading control. Values are mean±SEM.



Supplemental Figure VIII

TrkCd5 inhibits *Ad.NT-3*-induced neoangiogenesis in mouse ischemic muscles

Bar graph and microphotographs display the effect of TrkCd5 on *Ad.NT-3*-induced increase in capillary density (expressed as ratio of ischemic to contralateral number of capillaries per mm^2). Neovascularization was assessed after 2 weeks from the surgery. Black arrows point to capillaries. Scale bar= 40 µm. Values are mean±SEM, n=3 mice per group. ***P<0.005 *vs* vehicle.