Construction of AdsDll4 vector

Plasmid template containing full length human Dll4 was kindly provided by Dr. Richard Sainson (Weatherall Institute of Molecular Medicine, University of Oxford, UK). Primers targeting only the extracellular domain were designed to amplify 321-1901 nt of the mRNA sequence (Gene Bank accession number NM_019074). This extracellular domain was cloned into the pShuttle-CMV vector (AE51021, Qiagen) in which the sDll4 expression cassette was inserted into deleted E1 region of Adenovirus 5 genome. The forward primer contained SalI followed by the START codon of Dll4: 5' tta gtc gac ATG gcg gca gcg tcc cgg agc gcc t 3'. The reverse primer contained XbaI followed by the Stop codon of Dll4: 5' tga tct aga tta gaa gct ggg cgg caa gcc cac g 3'. The resulting recombinant vector was amplified and purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography, and finally plaque tittered in 293 cells as previously described.

Control adenovirus vectors

Ad-Null was constructed similarly to *Ad-sDll4*, but with no gene in the expression cassette was used as a control vector. Recombinant adenoviral vector encoding *Escherichia Coli* β-glactosidase gene driven by the CMV promoter was a kind gift of Graciela Sala-Newby, Bristol Heart Institute.

X-gal staining

Five days after gene transfer in ischemic side, the whole limb or dissected muscles, which were cut into 60μm sections, were stained with X-gal reaction as described previously.[1] Histological sections were counterstained with eosin.

Primers

Validated primers are purchased from Qiagen. Mouse Dll4 “QT01053598”, Human HES-1 “QT00039648”, mouse hes1 “QT00313537”, mouse nrarp1 “QT00262199”, mouse hey1 “QT00115094”, human rRNA 18S “QT00199367”, mouse rRNA 18S “QT01036875”, and human β-actin “QT01680476”.

Whole mount immunochemistry procedure

Mice were perfused under physiologic pressure with paraformaldehyde 4%PBS. Adductor muscles or hearts were isolated, carefully dissected under stereomicroscope and snap frozen. Frozen samples were cut into slices of 100μm thickness. Briefly, following incubation with blocking buffer, the samples were incubated with primary antibody: Goat polyclonal anti-mouse Dll4 (1:25, R&D systems), Rat monoclonal anti-mouse CD31 (1:25, BD Bioscience), Goat anti-mouse Podocalyxin (1:100, R&D systems), Rabbit anti-NG2 (1:20, Chemicon), Rat monoclonal anti-mouse CD45 (1:50, BD Bioscience) or Rat monoclonal anti-mouse CD11b (1:50, R&D system) overnight at 4°C. Further incubation with Isolectin B4-Alexa 568/488 (1:50, Invitrogen), anti-smooth muscle α-actin-cy3 antibody (1:100, Sigma), and Phaloidin-Alexa 488 (1:1000, Invitrogen) or/and appropriate secondary antibody was performed. Serial z-stack images of adductor muscle were generated using Leica SP5 AOBS confocal laser scanning microscope (Wolfson Bioimaging facility, University of Bristol).

To visualize vessel perfusion, anesthetized mice were injected intracardially with biotinylated GS-IsolectinB4 (100μg, invitrogen). After 5 min, muscles were fixed and subsequently labeled for injected lectin by incubating with streptavidin-Alexa 488. Co-staining is applied to visualize the total vessels by incubating with Isolectin B4-Alexa 568.[2] To reveal the effect of Dll4/Notch blockade on cell proliferation, Bromodeoxyuridine (BrdU) (Sigma-Aldrich) 10 mg/kg was administrated intraperitoneally 2 hours before sacrifice. Muscles were processed for whole-mount staining as described above. The staining for incorporated BrdU was performed as

described previously. DNA denaturation step was preceded by incubation with proteinase K and DNAase enzymes. Samples were then incubated overnight with Biotin conjugated anti-BrdU (1:50, Zymed), Isolectin B4-alexa 488 (1:50) and Rat anti-mouse CD45 (1:25) at 4°C. Next, samples were incubated with secondary antibody goat anti-rat alexa-568, Streptravidin-alexa 647 and DAPI for 4 hours at room temperature. Samples were mounted for microscopic analysis[3].

Oil O Red staining

After washing the fixed adductor muscle slices with PBS, slices were incubated in 60% Isopropanol for 5 min. Samples then were incubated in Oil O Red/60% Isopropanol solution for 20min. To remove excess of staining, samples were immersed in 60% Isopropanol and distilled water.

Image analysis and quantification

Local Connected Fractal Dimension analysis

Confocal images of network vessels of the adductor muscles whole-mount stained with endothelial cell marker (Pecam-1) were acquired with size 1550x1550x50 μm, 3 images per animal, n=5 per group. Next, confocal images were transformed in gray scale at resolution 320x320 pixels, processed to black/white binary conversion using Image J NIH software. Dimension fractal (D_f), a statistical descriptor of space-filling pattern and density, necessarily varies from 1 to 2 for 2D binary fractal images.[4] D_f was estimated for digitalized images with the software fracLac version 2.5, implementing the method of box counting. Local Connected Fractal Dimension (LFD) is a local dimension, calculated for each pixel in the same general way that a D_f for mass is calculated, using the slope of the log-log regression line for pixel mass against box size. Distribution of LFDs for an image represents the local variation in complexity of the whole vascular network of the sample.

Vessel network perfusion

Vascular perfusion quantification was performed on 3 fields per sample sized 1550x1550x70 μm, n=10 per group. Total muscle vasculature area-stained with Isolectin B4 (perfused+non-perfused vessels) and perfused vessel area-stained by *in vivo* intravascular injection of biotinylated lectin- were quantified by Image J NIH software. Perfused vessels were calculated as a percentage of perfused vessel area compared to total vascular area.

Fat degeneration and necrosis

Images were taken from three different levels of adductor muscles. Total muscle area, adipose tissue and necrotic area were measured by image J NIH software (n=6 per group).

Semi-quantification of capillary sprouts, leukocytes and proliferating cells

Quantification of vascular sprouts was performed in whole-mount of ischemic adductor muscles stained with Pecam-1. Capillary sprouts were defined as protrusive endothelial cells with filopodia extensions. Sprouts were counted in 5 fields (sized 455x455 μm, n=10 per group). Leukocytes (CD45-positive cells) and proliferating cells (BrdU positive cells) were counted from 3 fields per samples (sized 775x775x40 μm, n=10 per group). All quantifications were done using Image J NIH software.

Cell culture

Cultured Human Umbilical Endothelial Cells (HUVECs) were used to determine whether sDll4 produced by the adenovirus antagonizes Notch activation.

Briefly, we used transwell culture plates with polycarbonate membranes (3.0 µm pore size) (Costar) to perform endothelial cell co-culture. $2x10^5$ HUVECs were plated on the upper chambers, where they were infected with *Ad-null* or *Ad-sDll4* (50 pfu/cell). Twenty four hours after infection, other HUVECs ($3x10^5$ cells) were seeded on the lower chambers, which were coated with full length Dll4 (R&D systems) 1µg/mL to activate Notch signaling or BSA as a control and co-cultured with infected HUVECs for additional 24 hours. Next, cells in the lower chamber were harvested to isolate total RNA for real time PCR.

THP-1 human monocyte cell line were purchased from American Type Culture Collection and maintained in suspension in RPMI 1640 medium supplemented with 10%FBS. In all experiments, THP-1 cell line was differentiated into adherent monocyte/macrophage-like phenotype by incubation with 100 nM of phorbol-12-myristate-13-acetate (PMA, Sigma) for 24 hours. THP-1 were plated at density $1x10^6$ cell per well. 20µM of N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester (DAPT, Millipore), a gamma-secretase inhibitor was added to culture medium to block Notch signaling. Recombinant pro-inflammatory TNF-α (5ng/mL, R&D systems) was used to mimic inflammatory conditions. In some experiments, THP-1 were co-cultured with HUVECs previously transfected with *Ad-Null* or *Ad-sDll4* (100 pfu/cell) using transwell culture plates. $1x10^6$ THP-1 were plated on the lower chamber, which was coated with full length Dll4 or BSA as control and incubated with infected HUVECs for 24 hours. The supernatant was collected to measure IL-8 levels and THP-1 cells were harvested to isolate total RNA for real time PCR.

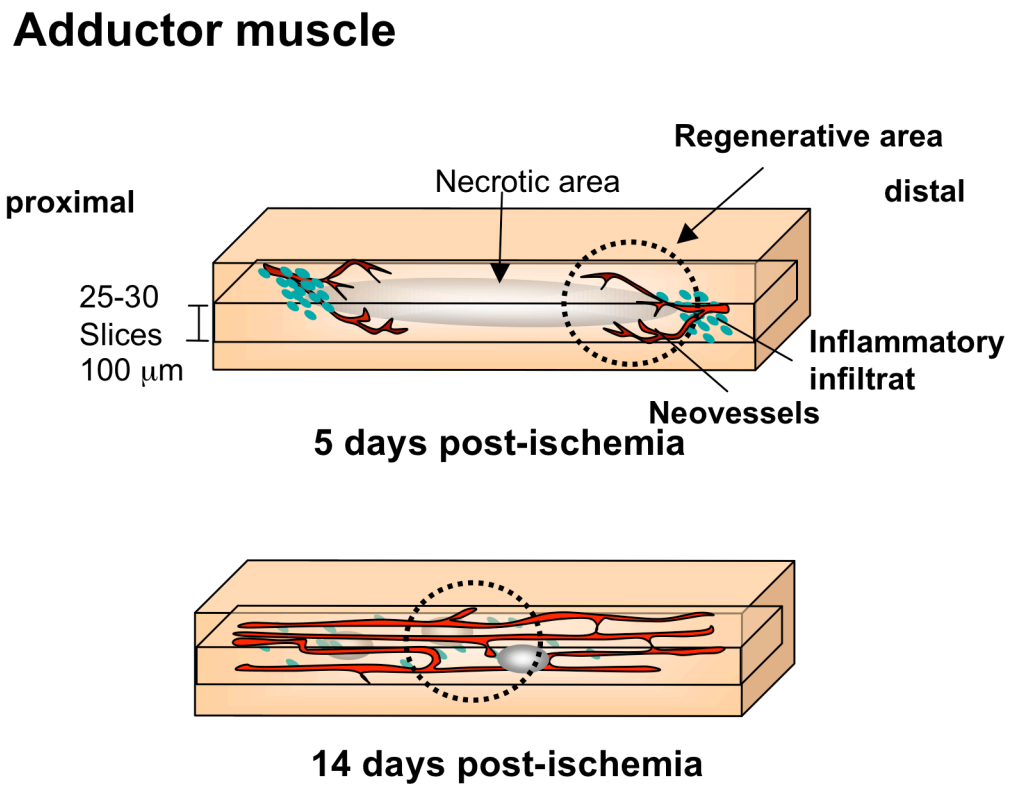
Matrigel assay

HUVECs were transfected with *Ad-sDll4* or *Ad-Null* (100 pfu/cell) 24 hours before assay. $4x10^4$ HUVECs were mixed with $6x10^4$ THP-1 monocytes, labeled with DiI (invitrogen). The cell mixture was plated on the matrigel using 4-wells plate (BD Bioscience). Phase-contrast time lapse videomicroscopy was used to record the process of endothelial cell network formation in the four conditions at the same occasion. Images were collected from 90min after plating on matrigel, for 4 hours at a rate of 1 image every 5 min, using an inverted Leica DMIRE2 microscope, with x10 phase-contrast objective, 12 bit monochrome CCD camera and Leica software. Cells were maintained at 37°C with 5% CO2 during image sequence acquisition.

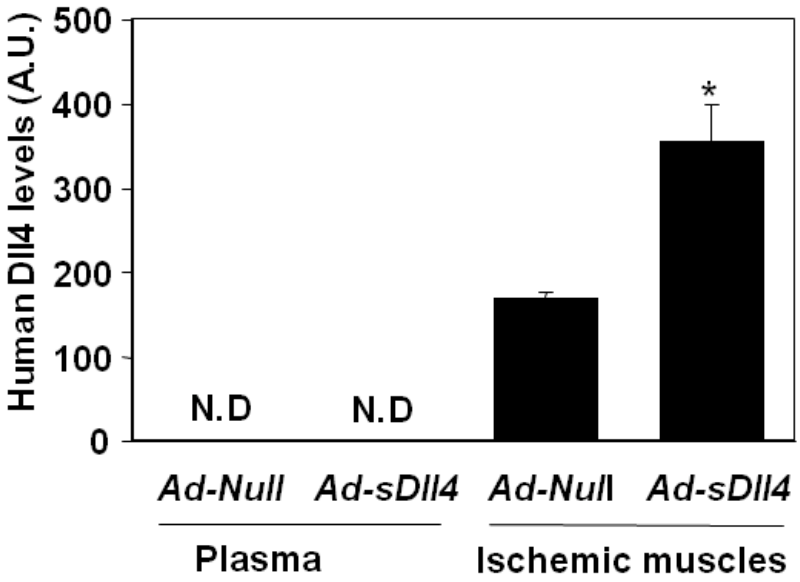
References:

1. Faerman A, Goldhamer DJ, Puzis R, Emerson CP, Jr., Shani M. The distal human myoD enhancer sequences direct unique muscle-specific patterns of lacZ expression during mouse development. *Dev Biol.* 1995;171:27-38.
2. Thurston G, Baluk P, Hirata A, McDonald DM. Permeability-related changes revealed at endothelial cell borders in inflamed venules by lectin binding. *Am J Physiol.* 1996;271:H2547-2562.
3. Tkatchenko AV. Whole-mount BrdU staining of proliferating cells by DNase treatment: application to postnatal mammalian retina. *Biotechniques.* 2006;40:29-30, 32
4. Parsons-Wingerter P, Elliott KE, Clark JI, Farr AG. Fibroblast growth factor-2 selectively stimulates angiogenesis of small vessels in arterial tree. *Arterioscler Thromb Vasc Biol.* 2000;20:1250-1256.

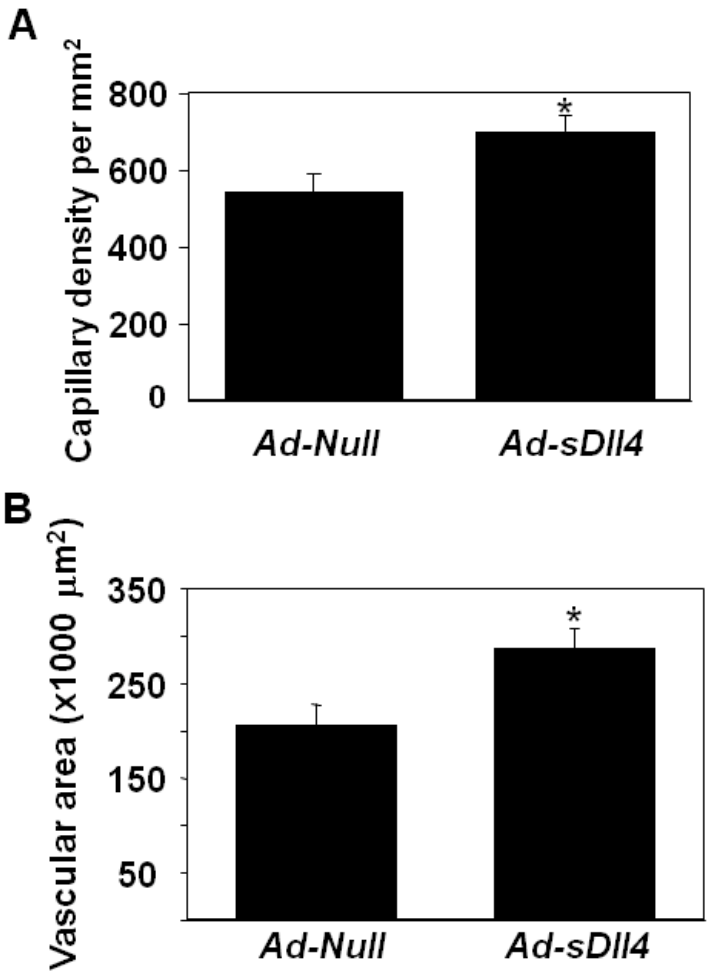
Online Figure I. Schematic representation of muscle neovascularization. The neovessels are shown at two distinct phases of tissue repair. The dotted line delimits the area of interest and the continuous line delimits the whole mounting slice examined by confocal microscopy. Early phase (5 days post-ischemia): capillary sprouting in peripheral regions of muscles. Late phase (14 days post-ischemia): new formed vascular network in muscle center region.



Online Figure II. Levels of soluble Dll4 in plasma and injected adductor muscles 3 days gene transfer, *P=0.04, n=4 per group. N.D. Not detectable.

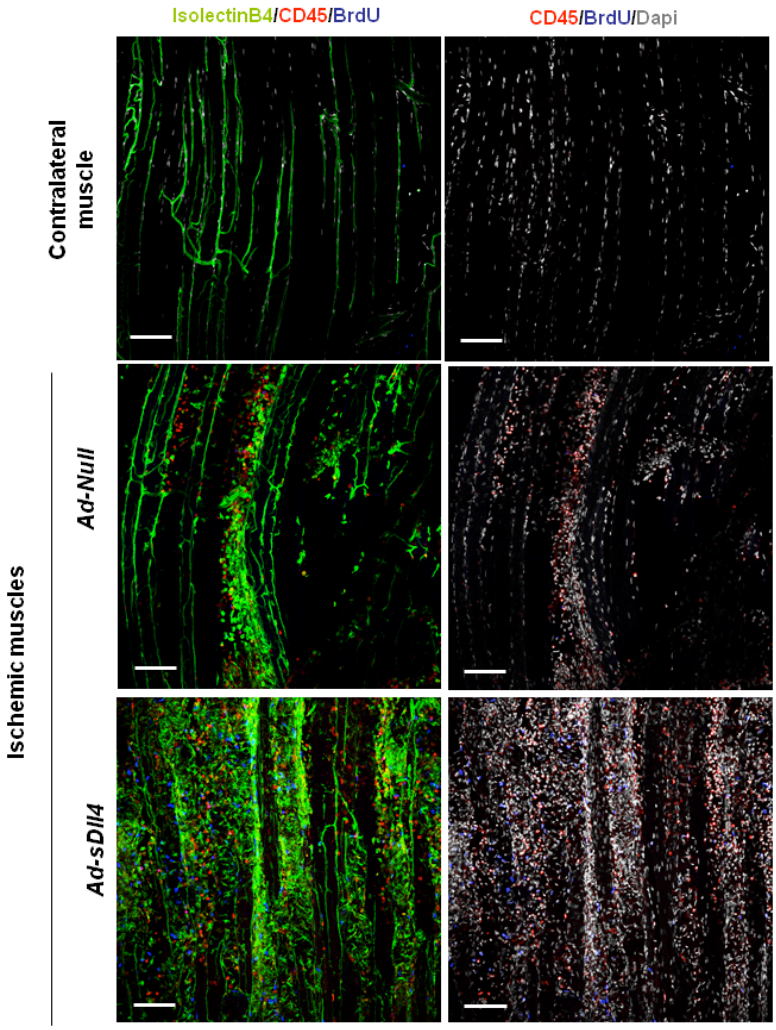


Online Figure III. Blockade of Dll4-mediated signaling increases total capillary density and vascular area in ischemic muscles at 14 days post-ischemia. A) IsolectinB4 positive capillaries were quantified on transverse section of *Ad-Null*- and *Ad-sDll4*-injected ischemic muscles. *P=0.01, n=12 per group. **B)** Total vascular area of capillary network was quantified on whole mount samples stained with IsolectinB4. *P=0.01, n=11.

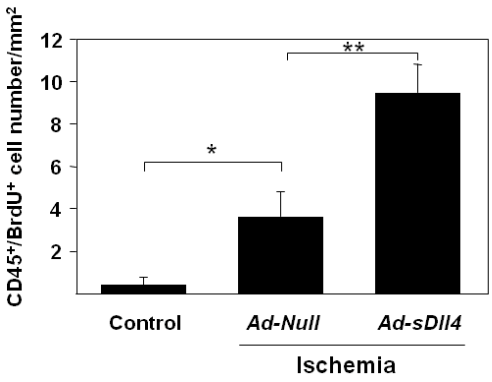


Online Figure IV. Inhibition of Dll4/Notch signaling increases proliferation of infiltrating leukocytes. A) Representative confocal images were analyzed 5 days post-ischemia: Isolectin B4 (green) and CD45 (red) and BrdU (Blue). Nuclei are revealed by Dapi (white/grey); scale bar: 100μm. **B)** The number of $CD45^+$ cells incorporating BrdU was increased in *Ad-sDll4*-injected ischemic muscles compared to *Ad-Null*-injected ischemic muscles. Control, normoperfused muscles. *P<0.05 and **P<0.01, n=6 each group.

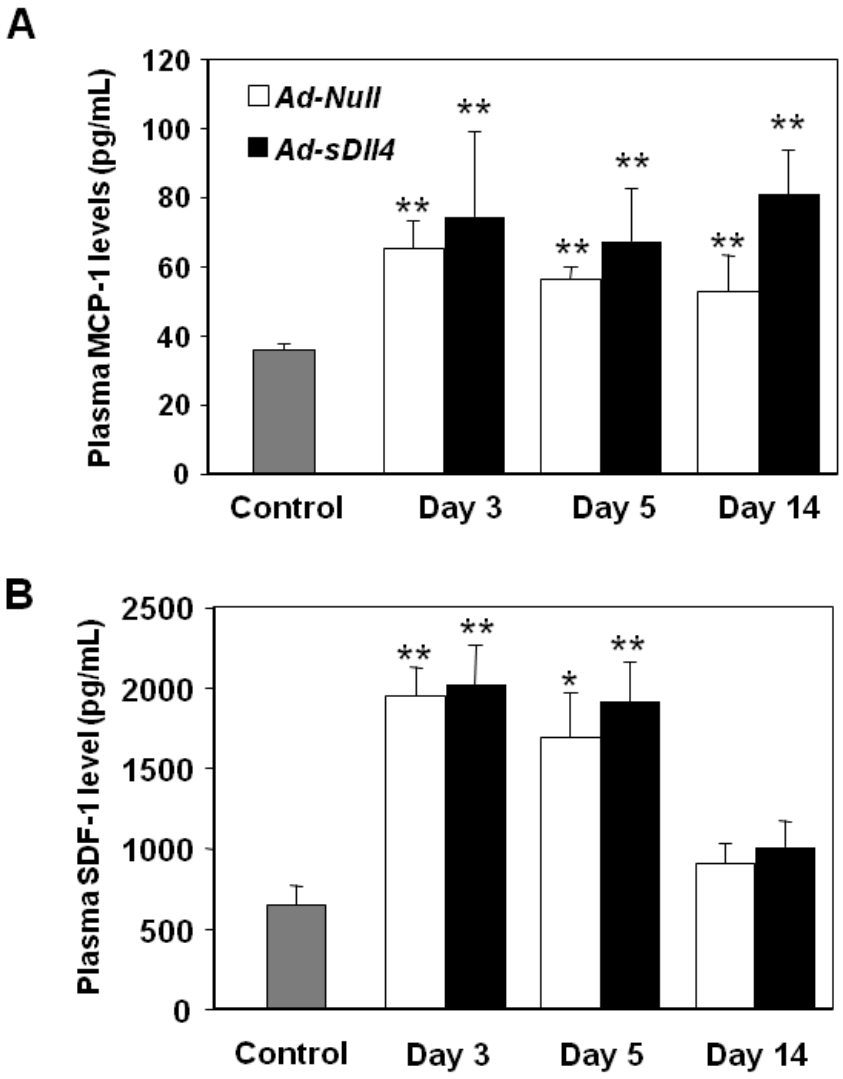
A



B



Online Figure V, Plasma of MCP-1 and SDF-1 measured by ELISA. *P<0.05, **P<0.01, ***P<0.001 vs. control; n=6 each group.



Online movie I, HUVEC infected with *Ad-Null* (100 pfu/cell). Time-lapse images were collected from 90 min after plating cells on Matrigel. Images were taken for 4 hours.

Online movie II, HUVEC infected with *Ad-sDll4* (100 pfu/cell). Time-lapse images were collected from 90 min after plating cells on Matrigel. Images were taken for 4 hours.

Online movie III, THP1 monocytes labelled with DiI (Red) were co-cultured with HUVEC infected with *Ad-Null* (100 pfu/cell). Time-lapse images were collected from 90 min after plating cells on Matrigel. Images were taken for 4 hours.

Online movie IV, THP1 monocytes labelled with DiI (Red) were co-cultured with HUVEC infected with *Ad-sDll4* (100 pfu/cell). Time-lapse images were collected from 90 min after plating cells on Matrigel. Images were taken for 4 hours