

Vascular Endothelial Growth Factor Overexpression Does Not Enhance Adipose Stromal Cell–Induced Protection on Muscle Damage in Critical Limb Ischemia

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Objectives—Critical limb ischemia complicates peripheral artery disease leading to tissue damage and amputation. We hypothesized that modifying adipose stromal cells (ASCs) to overexpress human vascular endothelial growth factor 165 (VEGF) would limit ischemic muscle damage to a larger extent than nonmodified ASCs.

Approach and Results—Rabbits with critical hindlimb ischemia were injected with allogeneic abdominal fat-derived ASCs transfected with plasmid-VEGF₁₆₅ (ASCs-VEGF; n=10). Additional rabbits received nontransfected ASCs (ASCs; n=10) or vehicle (placebo; n=10). One month later, ASCs-VEGF rabbits exhibited significantly higher density of angiographically visible collaterals and capillaries versus placebo (both $P<0.05$) but not versus ASCs (both $P=NS$). Arteriolar density, however, was increased in both ASCs and ASCs-VEGF groups (both $P<0.05$ versus placebo). ASCs-VEGF and ASCs showed comparable post-treatment improvements in Doppler-assessed peak systolic velocity, blood pressure ratio, and resistance index. Ischemic lesions were found in 40% of the muscle samples in the placebo group, 19% in the ASCs-VEGF group, and 17% in the ASCs groups (both $P<0.05$ versus placebo, Fisher test).

Conclusions—In a rabbit model of critical limb ischemia, intramuscular injection of ASCs genetically modified to overexpress VEGF increase angiographically visible collaterals and capillary density. However, both modified and nonmodified ASCs increase arteriolar density to a similar extent and afford equal protection against ischemia-induced muscle lesions. These results indicate that modifying ASCs to overexpress VEGF does not enhance the protective effect of ASCs, and that arteriolar proliferation plays a pivotal role in limiting the irreversible tissue damage of critical limb ischemia. (*Arterioscler Thromb Vasc Biol.* 2015;35:184-188. DOI: 10.1161/ATVBAHA.114.304348.)

Key Words: adult somatic stem cells ■ angiogenesis effect ■ vascular endothelial growth factor A

Peripheral arterial disease is caused by atherosclerosis of peripheral arteries. Its progression leads to critical limb ischemia, a condition with estimated 25% mortality in the first year.¹ This mortality rate is even higher in patients requiring amputation.¹ Despite the new advances in revascularization therapies, treatment is not effective in most cases.² Therefore, angioarteriogenic strategies aimed at restoring blood flow in the ischemic limb through cell and gene therapy have emerged as possible alternatives.

Previous studies have shown that adipose stromal cells (ASCs) have a marked angiogenic potential in vivo, improving tissue perfusion in animal models of hindlimb ischemia mainly through the release of multiple angiogenesis-related growth factors and cytokines.³⁻⁵ Moreover, recent phase I trials in patients with critical limb ischemia showed that angiogenic treatments with autologous ASCs are safe and potentially beneficial.⁶⁻⁸

On the other hand, we have shown that direct intramuscular injection of a plasmid encoding human vascular

endothelial growth factor 165 (VEGF₁₆₅) in rabbits with hindlimb ischemia increases microvascular density. However, protection against ischemic muscle lesions in the affected limb was afforded only if the treatment was repeated, the reason being that VEGF expression, and hence the vasculogenic stimulus, decayed steeply after the seventh day post injection.⁹

Therefore, we hypothesized that genetically modifying ASCs to overexpress human VEGF₁₆₅ would enhance the vasculogenic potential of ASCs, not only by increasing the paracrine angiogenic effect of ASCs but also by attenuating the decay of VEGF expression in the target tissue. In turn, these effects would protect the ischemic muscle against necrotic lesions to a significantly larger extent than nontransfected ASCs.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

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Nonstandard Abbreviations and Acronyms

ASCs	adipose stromal cells
ASCs-VEGF	ASCs transfected with a plasmid encoding vascular endothelial growth factor 165
PSV	peak systolic flow velocity

Results

VEGF-Overexpressing ASCs Retention in the Target Tissue Is High at 7 Days and Low at 30 Days After Implantation

ASCs taken from the abdominal fat were positive for the anti-CD44 antibody, mildly positive for the anti-CD90 and anti-CD105 antibodies, and negative for anti-CD45 and anti-CD34 antibodies. ASCs were able to differentiate into osteocyte and chondrocyte lineages (Figure I in the online-only Data Supplement).

Before performing the main protocol, we aimed at studying retention and viability of the study cells in the target tissue. To this end, ASCs from male donors were transfected with a plasmid encoding human VEGF₁₆₅ (ASCs-VEGF), stained with a fluorescent dye and injected in ischemic left adductor and quadriceps muscles of female recipients. Fluorescence microscopy revealed that injected ASCs-VEGF were present in considerable amount at 7 days and scarcely at 30 days (Figure 1A and 1B). Assessment of the sex-determining region Y gene (SRY) of the male donor ASCs followed the same pattern, namely high amplification at 7 days (616.1±240.1-fold increase with regard to control) and low at 30 days (12.5±14.9; Figure 1C).

Transgene Expression Decreases Over Time but Is Still Detectable at 30 Days After Treatment

Transgene expression in the target muscles was assessed by reverse transcriptase-polymerase chain reaction (Figure 1D). Human VEGF mRNA was maximum at 7 days (11 160.8±818.9-fold increase versus control) and decreased at 14 (10.7±13.1), 21 (2.8±1.3), and 30 (2.2±1.7) days. Immunohistochemistry against human VEGF in injected muscles of ASCs-VEGF-treated

rabbits showed VEGF protein expression at 7 and 30 days post injection. Slices from a placebo-treated rabbit at the same time points are also depicted (Figure 1E).

VEGF-Transfected ASCs and Nontransfected ASCs Induce Comparable Improvements in Ischemic Limb Hemodynamics

Rabbits undergoing full extirpation of the left superficial femoral artery and ligation of the deep left femoral artery were injected in the target muscles with ASCs (n=10), ASCs-VEGF (n=10), or vehicle (n=10). One and 30 days after surgery, we performed a Doppler echo on both hind limbs to assess pre- and post-treatment hemodynamic variables (Figure 2).

Peak systolic flow velocity (PSV) increased significantly in ASCs-VEGF group from 11.1±3.3 cm/s (mean±SD) to 23.4±19 cm/s at 30 days ($P<0.05$). In the ASCs and placebo groups, PSV showed a tendency to increase but no statistically significant differences were found (Figure 2A). The ratio of PSV of the ischemic limb to PSV of the nonischemic limb did not change in the placebo group (0.21±0.13–0.51±0.46; $P=NS$) but improved in both treated groups (ASCs, from 0.32±0.24 to 0.72±0.4; $P<0.05$ and ASCs-VEGF, from 0.28±0.16 to 0.7±0.57; $P<0.05$; Figure 2B). The ratio of PSV to diastolic flow velocity did not change significantly in the placebo group (from 1.79±0.53 to 2.42±0.98; $P=NS$) but increased both in the ASCs group (from 1.79±0.44 to 3.55±2; $P<0.05$) and in the ASCs-VEGF group (from 1.89±0.34 to 3.77±1.63; $P<0.01$; Figure 2C). A similar behavior exhibited the resistance index, which did not vary in placebo animals (0.37±0.19–0.48±0.31; $P=NS$) and increased in ASCs (0.42±0.13–0.64±0.17; $P<0.05$) and ASCs-VEGF (0.45±0.1–0.69±0.12; $P<0.01$) groups (Figure 2D).

The blood pressure ratio increased significantly in the ASCs group (0.29±0.27–0.63±0.23; $P<0.05$) and showed a strong tendency to increase (yet not statistically significant) in the ASCs-VEGF group (from 0.26±0.14 to 0.59±0.28; $P=NS$). No significant change in blood pressure ratio was observed in the placebo group (from 0.39±0.22 to 0.54±0.27; $P=NS$). Overall, these results show that both treated groups exhibited comparable over time improvements in ischemic limb hemodynamics.

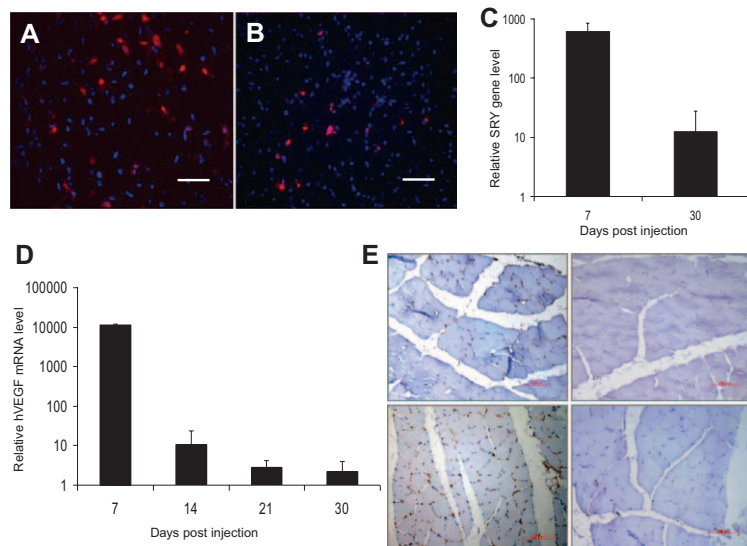


Figure 1. Cell tracking and transgene expression. Male vascular endothelial growth factor (VEGF)-overexpressing adipose stromal cells stained red with fluorescent dye PKH26 were present in the ischemic muscle of female recipients at 7 days post injection (A) and in lesser amount at 30 days post injection (B); nuclei are stained blue. C, Amplification of the sex-determining region Y gene (SRY) of male donor adipose stromal cells in the ischemic muscle of female recipient confirmed presence of viable injected cells at 7 and 30 days post injection. D, Reverse transcriptase-polymerase chain reaction analysis of ischemic muscle injected with VEGF-overexpressing adipose stromal cells showed human VEGF (hVEGF) expression up to ≥30 days after cell injection. Mean±SD are depicted. E, Immunohistochemistry against human VEGF protein (brown staining) in a rabbit treated with VEGF-transfected adipose stromal cells at 7 (upper left) and 30 (lower left) days after treatment, and a placebo-treated rabbit at 7 (upper right) and 30 (lower right) days after treatment. Bars, 100 μm.

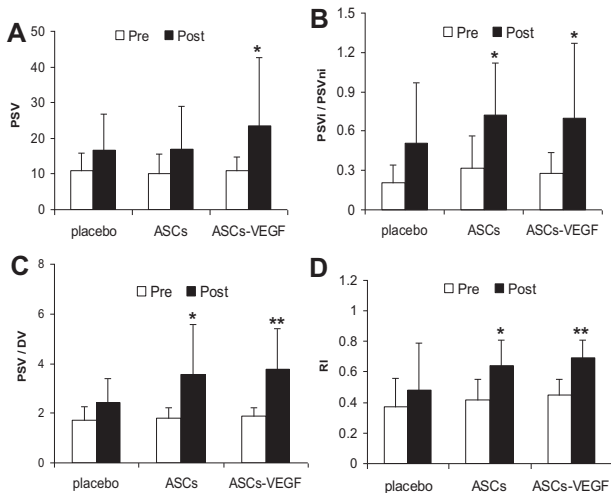


Figure 2. Hemodynamic variables assessed by Doppler echo before (pre) and 30 days post-treatment (post). **A**, Peak systolic flow velocity (PSV) improved significantly in the group treated with vascular endothelial growth factor (VEGF)-overexpressing adipose stromal cells (ASCs-VEGF). **B–D**, The ratio of PSV of the ischemic limb (PSVi) to PSV of the non ischemic limb (PSVni), the ratio of PSV to diastolic flow velocity (DV) of the ischemic limb, and the resistance index (RI) improved significantly in the groups receiving modified and nonmodified ASCs. **P*<0.05; ***P*<0.01 vs pretreatment (ANOVA-Bonferroni). Values are mean±SD.

VEGF-Overexpressing ASCs Enhance Angiographically Visible Collateralization

Thirty days after treatment, immediately before euthanasia, angiography was performed to assess for collateralization in the ischemic limb. The ASCs-VEGF group had higher collateral density (5.6±1.1 vessels/cm²) than placebo group (4.5±0.6 vessels/cm²; *P*<0.04). Collateral density of ASCs group (4.8±1.1 vessels/cm²) did not differ (*P*=NS) from placebo (Figure 3).

VEGF-Transfected ASCs Increase Capillary Density More Than ASCs, but Both Treatments Increase Arteriolar Density Similarly

At 30 days post treatment, ASCs-VEGF group showed significantly increased capillary density (100.2±40.1 capillaries/100 myocytes) compared with placebo (50.3±20.3; *P*<0.05). The capillary density found in ASCs group (89.2±22.8) did not differ significantly from that found in placebo (Figure 4A).

Both ASCs-VEGF (11.8±2.9 arterioles/mm²) and ASCs (10.8±3.7 arterioles/mm²) groups increased arteriolar density

at 30 days post treatment compared with placebo (5.9±2.2; *P*<0.05 versus ASCs and ASCs-VEGF; Figure 4B).

ASCs and VEGF-Transfected ASCs Afford Equal Protection Against Ischemic Muscle Damage

Samples obtained from the ischemic adductor and quadriceps were assessed for ischemic lesions under light microscopy. Three types of ischemic muscle lesions were observed: (1) decreased density of fibers plus adipose tissue replacement; (2) atrophy and lipomatosis; and (3) areas of necrosis, fiber replacement with scar tissue, and myositis.

Independent from the type of lesion, samples were divided into normal and pathological and expressed as percentage of the total samples. The percentage of samples displaying pathological muscle lesions was almost equally lower in ASCs-VEGF (19%) and ASCs (17%) when compared with placebo (40%; *P*<0.05 versus ASCs and ASCs-VEGF; Fisher test; Figure 5).

Discussion

Our results show that VEGF-overexpressing ASCs induced a higher density of angiographically visible collaterals and capillaries. However, their effect on protection against ischemic muscle lesions was equal to that afforded by nontransfected ASCs. Both treatments increased arteriolar density to a similar extent, suggesting that tissue protection depended principally on the development of a network of smooth muscle-walled microvessels. ASCs-induced arteriolar proliferation was to be expected, on account that they secrete several cytokines and growth factors, other than VEGF, involved in vasculogenesis, such as hepatocyte growth factor, transforming growth factor-β, fibroblast growth factor-2, insulin like growth factor-3, and stromal cell-derived factor-1.^{3,10,11} Given the well-known angiogenic effect of VEGF, it is also sound that the group treated with ASCs that overexpressed VEGF exhibited higher capillary density.

Given that in terms of capillary and collateral densities, the differences between ASCs-VEGF and ASCs did not achieve significance, it is reasonable to assume that these neovessels participated in the protective effect. However, the arteriolar density results underscore the importance of arteriolar neof ormation in treatments directed to restore muscle perfusion and limit ischemic damage, as has been previously claimed.¹² Besides being more stable and less prone to regress than neovessels formed by only endothelium, arterioles respond to the physiological stimuli regulating vascular tone and thus

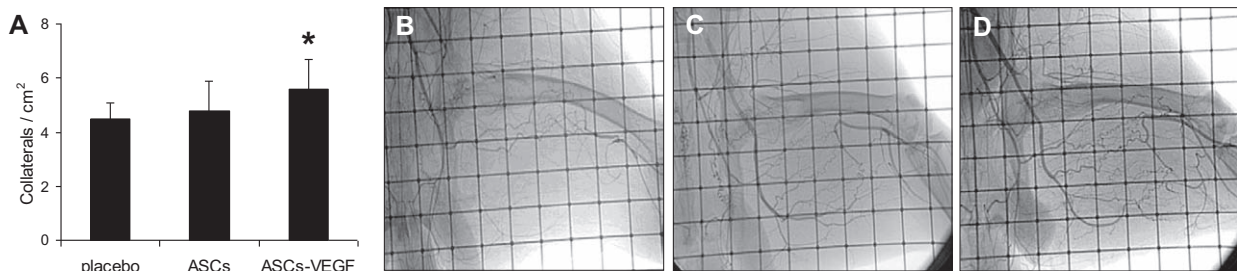


Figure 3. Angiography at 30 days post treatment. **A**, Density of angiographically visible collaterals was significantly higher vs placebo in the group treated with vascular endothelial growth factor (VEGF)-overexpressing adipose stromal cells (ASCs-VEGF). Examples of angiography images of a placebo-treated rabbit (**B**), a rabbit treated with nonmodified adipose stromal cells (**C**), and one treated with VEGF-overexpressing ASCs (**D**). **P*<0.05 vs placebo (ANOVA-Bonferroni). Values are mean±SD.

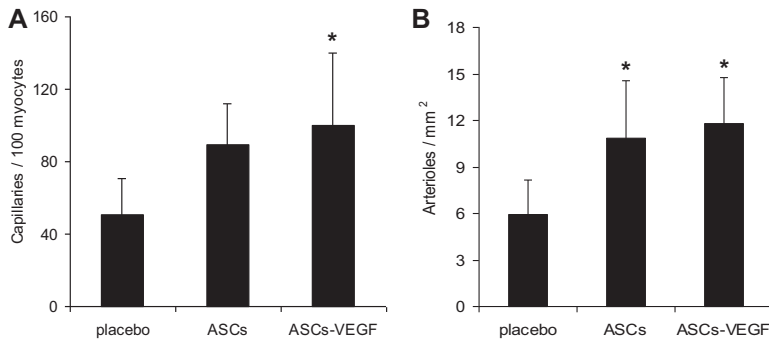


Figure 4. Microvascular proliferation. At 30 days post treatment, capillary density (**A**) was significantly higher than placebo in the group treated with vascular endothelial growth factor (VEGF)-overexpressing adipose stromal cells (ASCs-VEGF). Arteriolar density (**B**) was significantly higher than placebo in the ASCs-VEGF group and in the group treated with nonmodified adipose stromal cells (ASCs). * $P < 0.05$ vs placebo (ANOVA-Bonferroni). Values are mean \pm SD.

tissue perfusion. Capillaries, however, permit the exchange of gases and nutrients between blood and cells, but their blood flow critically depends on an arteriolar network adequate in size and function. It is, therefore, not surprising that both treatments exerted similar protection against ischemic muscle lesions.

As was the case with capillaries, the higher collateral vessel density observed in the ASCs-VEGF group made no difference in terms of muscle protection when compared with the nontransfected ASCs group. Moreover, the influence of a higher number of angiographically visible collaterals on the diverse variables assessed by Doppler echo was subtle. In effect, in the ASCs-VEGF group, the only parameter in which the post-treatment improvements achieved statistical significance was PSV. The ratio of PSV of ischemic to nonischemic limb, as well as the PSV/diastolic velocity index and the resistance index, improved similarly in both treated group. Furthermore, the post-treatment improvement observed in blood pressure ratio achieved significance in the group treated with nontransfected ASCs. Taken together, these results help concluding that tissue salvage depended principally on arteriolar proliferation.

Previous studies have shown that the neovessels induced by angiogenic treatments lack stability and tend to regress in the absence of the mitogenic stimulus.^{13,14} According to Dor et al,¹⁵ in the case of VEGF, the critical time for the neovessels to become refractory to VEGF withdrawal is 4 weeks. In our rabbits, we tracked the injected VEGF-transfected ASCs by visualization of stained cells and detection of the Y gene of male donor cells in female recipients and found that cell retention and viability were low but still detectable at 1 month after injection. Human VEGF expression followed a similar pattern, decreasing over time but lasting (although in low level) until the end of follow-up. These results indicate that a certain degree of paracrine mitogenic stimulation persisted beyond the period considered necessary for the stabilization of the neovascular network. In effect, human VEGF protein was readily observed in injected muscles of ASCs-VEGF-treated rabbits at 30 days after treatment. However, in terms of muscle protection, our results did not support the hypothesis that VEGF overexpression would enhance the benefits of ASCs implant in ischemic limbs. In this regard, our results differ from those of Shevchenko et al,¹⁶ who implanted 5×10^5 human ASCs transduced with an adenoassociated virus encoding VEGF₁₆₅ in nude mice with hindlimb ischemia. These authors found significantly higher arteriolar density and significantly lower muscle damage in mice treated with transduced ASCs. However, the results are not strictly comparable, not only because of differences in the donor cells (human versus canine) and the host species (nude mouse versus wild-type rabbit) but also because of the vector used. The authors do not report the transduction efficiency reached with the adenoassociated virus, but it is reasonable to assume that it was high. In our experiments, the maximum transfection efficiency achieved after optimizing the relationship between transfection reagent and DNA mass was 20%, similar to the value reported by Gheisari et al¹⁷ for rat bone marrow mesenchymal stromal cells. Therefore, the amount of VEGF-overexpressing ASCs injected was ≈ 2 million in our rabbits and 5×10^5 in Shevchenko's mice. Considering the size of both animals, the concentration of VEGF-overexpressing cells in the target tissue was ≥ 5 -fold higher in their mice, a fact that may account for the differences observed.

In conclusion, allogeneic ASCs overexpressing human VEGF₁₆₅ increase capillary density and angiographically visible collaterals in a rabbit model of hindlimb ischemia. However, they afford equal protection against ischemic muscle lesions than nontransfected ASCs, because of the fact that both treatments increase arteriolar density to a similar extent.

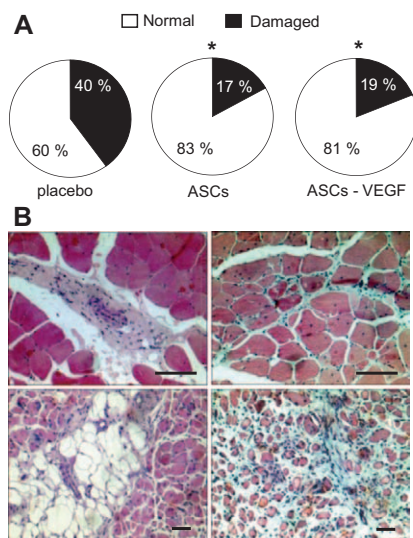


Figure 5. Ischemic muscle damage. **A**, Percent muscle samples showing ischemic lesions was significantly lower both in the group treated with vascular endothelial growth factor (VEGF)-overexpressing adipose stromal cells (ASCs-VEGF) and nonmodified ASCs when compared with placebo. **B**, Histology samples of ischemic muscle showing the types of lesions found. Upper left, normal; upper right: decreased density of fibers plus mild interstitial fibrosis; lower left: atrophy and lipomatosis; and lower right: areas of necrosis, fiber replacement with scar tissue and myositis. * $P < 0.05$ (Fisher test). Bars, 100 μ m.

These results indicate that arteriolar neof ormation plays a pivotal role for tissue salvage in critical limb ischemia.

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Disclosures

None.

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Significance

To enhance their regenerative potential, stem cells are usually modified to overexpress angiogenic growth factors. In rabbits with critical limb ischemia, we show that allogeneic adipose stromal cells modified to overexpress human vascular endothelial growth factor do not afford greater protection against ischemic muscle damage than nonmodified cells, despite the fact that modified cells induce larger capillary growth and angiographically visible collaterals than nontransfected cells. The reason for modified and nonmodified adipose stromal cells exhibiting similar anti-ischemic protective effect seems to be their equal ability to induce arteriolar growth, highlighting the pivotal role of arteriolar neof ormation for tissue salvage in critical limb ischemia.

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

All procedures were done in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication N° 85-23, revised 1996) and approved by the Laboratory Animal Care and Use Committee of the Favaloro University.

Cell isolation and culture

Rabbits were anesthetized (intravenous xylazine 7 mg/kg and ketamine 25 mg/kg) and adipose tissue was isolated from the abdominal region. Cell isolation was performed according to Van der Bogt et al.¹ Briefly, the tissue was digested with 0.05% collagenase (type IV, Sigma Aldrich, St Louis, MO, USA). Cells were cultured at 37°C and 5% CO₂ with low glucose DMEM (Gibco, Grand Island, NY, USA), 20% FBS (Internegocios S.A., Argentina) and Antibiotic-Antimycotic (Gibco). After 3 days the complete medium was replaced. Adherent cells were expanded until 80% confluence and passage 4.

Cell identification

Cells were characterized by their surface antigenic profile using FITC-conjugated anti-CD44 and CD45 antibodies (AbD Serotec, Oxford, UK), and PE-conjugated anti-CD90 CD105 and CD34 antibodies (BD Biosciences, Franklin Lakes, NJ, USA). Each sample was analyzed by flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA) and Cyflogic 1.2.1 software (Perttu Terho & Cyflo, Turku, Finland). Osteogenic and chondrogenic differentiation was induced to assess for cell plasticity using the appropriate STEM PRO differentiation kits (Gibco) and stains (Alizarin Red S and Alcian Blue 8 GX, Sigma-Aldrich, St Louis, MO, USA), respectively (Suppl. Figure I).

Cell transfection

We intended to maximize transfection efficiency by testing diverse combinations of commercially available transfection reagents and masses of a plasmid encoding the

green fluorescent protein gene (pGFP, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Flow cytometry analysis showed that maximum transfection efficiency (20%) was achieved with 3 μ l of Lipofectamine 2000 (Gibco) per 1 μ g DNA in 4 cm² culture area at 80% confluence.

Using this protocol, ASCs were transfected with a plasmid encoding human VEGF₁₆₅ (ASCs-VEGF). The eukaryotic expression vector (pVEGF₁₆₅) is a 3930 bp plasmid that includes the human VEGF₁₆₅ coding gene, transcriptionally regulated by the cytomegalovirus promoter/enhancer. A SV40 terminator is located 3' to the VEGF₁₆₅ coding gene. Preparation, purification and quality control analyses of the plasmid from transformed Escherichia Coli cultures were performed under GMP conditions (Biosidus, Buenos Aires, Argentina).

Surgical preparation and treatment

Thirty male and 8 female rabbits of approximately 3.4 \pm 0.62 kg weight were anesthetized with xylazine (7 mg/kg) and ketamine (25 mg/kg), and maintained with intravenous 0.1 ml injections of xylazine-ketamine (1:9 dilution) every 3 minutes. Topical lidocaine hydrochloride 2 g% was also administered. All animals spontaneously breathed oxygen-enriched room air during surgery. Heart rate and arterial oxygen saturation were monitored with a pulse oxymeter (Novamatrix 515A, Wallingford, CT, USA). The 30 male rabbits underwent the protocol testing therapeutic efficacy, whereas the 8 female animals were used for cell tracking and transgene expression assessment.

The animal model has been described elsewhere.² Briefly, a longitudinal incision in the left hindlimb was performed to entirely excise the superficial femoral artery from its origin up to its bifurcation into the saphenous and popliteal arteries. The deep femoral artery was ligated at its origin and left in place. Finally, the surgical wound was closed and a single prophylactic dose of cephalaxine 50 mg/kg was administered intravenously.

One day later, the 30 animals of the efficacy protocol were randomized to receive the following treatments: 2 ml of PBS containing 10⁷ ASCs transfected with pVEGF₁₆₅ (ASCs-VEGF group, n=10), 10⁷ non-transfected ASCs (ASCs group, n=10) or PBS (placebo group, n=10). The therapy was delivered in 10 injections distributed in the quadriceps (4 injections) and the adductor muscles (6 injections). The nature of the injectates was kept blind for all investigators until the end of data analysis. All 8 female rabbits of the cell tracking and transgene expression protocol were injected in the ischemic adductor and quadriceps muscles with 10⁷ ASCs-VEGF cells from a male

donor. Cells were marked with the red fluorescent PKH26 stain (Sigma Aldrich). Rabbits were sacrificed at 1, 2, 3 and 4 weeks post injection (n=2 for each time point). Muscle samples from the injected zone were collected and frozen to detect cell viability by fluorescence microscopy (red stained cells) and by qPCR detection of sex-determining region Y gene (SRY) of the male donor. Human VEGF transgene expression in the muscle samples was assessed by RT-qPCR (see below).

Doppler Echography

Prior to the treatment procedure and 30 days after treatment Doppler echography was performed to measure peak systolic flow velocity (PSV) and diastolic velocity (DV) of both hind limbs. Pulsed Doppler ultrasound and a high-definition 5-12 MHz linear transducer (Philips ATL 5000, Bothell, WA, USA) were used. The color Doppler device of the equipment was used to identify the artery. Care was taken to use a 60° angle and the least sample volume.

The systolic/diastolic ratio (S/D) was calculated as PSV/DV , the resistance index (RI, also referred to as the Pourcelot ratio) was calculated according to the equation: $RI=(PSV-DV)/PSV$ and the ratio between PSV of the ischemic (i) and non ischemic (ni) hind limbs was calculated as PSV_i/PSV_{ni} .³

Systolic pressure was determined using an inflatable cuff 9 cm in length and 2 cm in width (Hokanson, Bellevue, WA, USA) positioned around the thigh. The cuff was inflated beyond peak systolic pressure (Hokanson E20 Rapid Cuff Inflator & AG101 Air Source), and slowly deflated until an audible signal was detected (Parks 811 Pulsed Doppler Ultrasound Detector and standard 10 MHz pencil probe, Aloha, OR, USA). The procedure was repeated 3 times with 5 minutes intervals and systolic blood pressure was considered to be the average of the 3 determinations. This value was used to calculate the blood pressure ratio, defined as the ratio of systolic pressure (in mm Hg) in the ischemic left hind limb to systolic pressure (in mm Hg) in the normoperfused right hind limb.

Angiography

At 30 days post treatment (just prior to euthanasia) the rabbits were anesthetized and an internal iliac artery angiography was performed. A 5F sheath was introduced in the aortic bifurcation by visually exposing the abdominal aorta. Two injections of 20 ml of iopamidol were administrated (10 ml per second) at 450 psi using an injector (Medrad Mark V, Medrad Inc. Indianola, USA). Images were obtained with DSA technique in 9

inches fields at 2.5 frames per second with an angiographer (Philips Integris 3000, Philips Medical System BV, The Netherlands). A squared grid was positioned between the rabbit and the table to count collateral vessels per cm^2 by two operators who were blinded to the treatment received. At the end of the study the animals were euthanized by a propofol overdose.

Histology and immunohistochemistry

Immediately after sacrifice, ischemic muscle samples were obtained and fixed in 10% formaldehyde for histological analysis. Samples were cut in halves and embedded in paraffin. Sections measuring 5 μm thickness were stained with Masson's trichrome to evaluate percentage of pathological muscle lesions.

One independent observer blindly analyzed the samples and characterized the lesions. The muscle samples were classified as normal or pathologic (independently from the kind of lesion found), and the number of pathological samples in each group were expressed as a percent of the total samples analyzed.

Capillaries and arterioles were detected by immunohistochemistry. For capillary detection, ischemic muscle sections were incubated with Biotinylated Euonymus Europaeus Lectin⁴ (Vector Laboratories, USA) and revealed using peroxidase-labeled streptavidin with AEC as chromogen. Sections were counterstained with hematoxylin. Capillary density was expressed as number of capillaries/100 myocytes in 20 photographs taken randomly of whole sections at 20x magnification.

Arterioles were detected using anti- α -smooth muscle actin (Bio Genex, San Ramon, CA, USA). Arteriolar density was expressed as number of arterioles measuring 8 to 50 μm in diameter/ mm^2 in whole sections. Quantification was done using an image analysis software (Image Pro Plus, Media Systems Corp, Silver Spring, USA).

Human VEGF protein in muscle samples of ASC-VEGF-treated rabbits was detected by immunohistochemistry using an anti-human VEGF antibody (Bio Genex).

Cell tracking

Frozen samples taken from the ischemic muscles of the 8 females injected with male ASCs-VEGF were pulverized in liquid nitrogen to obtain DNA and RNA. Total DNA was isolated (Qiagen, Hilden, Germany) and quantitated (A260 nm spectrophotometry).

Real time PCR amplification was performed using SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY, USA) and rabbit SRY gene specific primers (Forward: 5'-GCA ATA CAG GAG GAA CAC GTA AAG T-3' and Reverse: 5'-CGT TCA TGG

GTC GCT TGA C-3') designed with primer express 3.0 software (Applied Biosystems, Foster City, CA, USA).

Human VEGF transgene expression

Total RNA was isolated (Trizol reagent, Gibco BRL, Grand Island, NY, USA), treated with DNase I (Promega, Madison, WI, USA), quantitated and reverse-transcribed (Improm-II™ reverse transcriptase protocol, Promega). Real time PCR reactions were carried out using human VEGF gene specific primers (Forward: 5'-ACG TAC TTG CAG ATG TGA CAA G-3' and Reverse: 5'-GTG GCG GCC GCT CTA-3'). In each reaction, GAPDH gene amplification was performed as endogenous control using specific primers (Forward: 5'-GGT CGT CTC CTG CGA CTT CA-3' and Reverse: 5'-GCC CCA GCA TCG AAG GT-3'). Data were processed to obtain a relative quantification analysis ($\Delta\Delta ct$).

Statistics

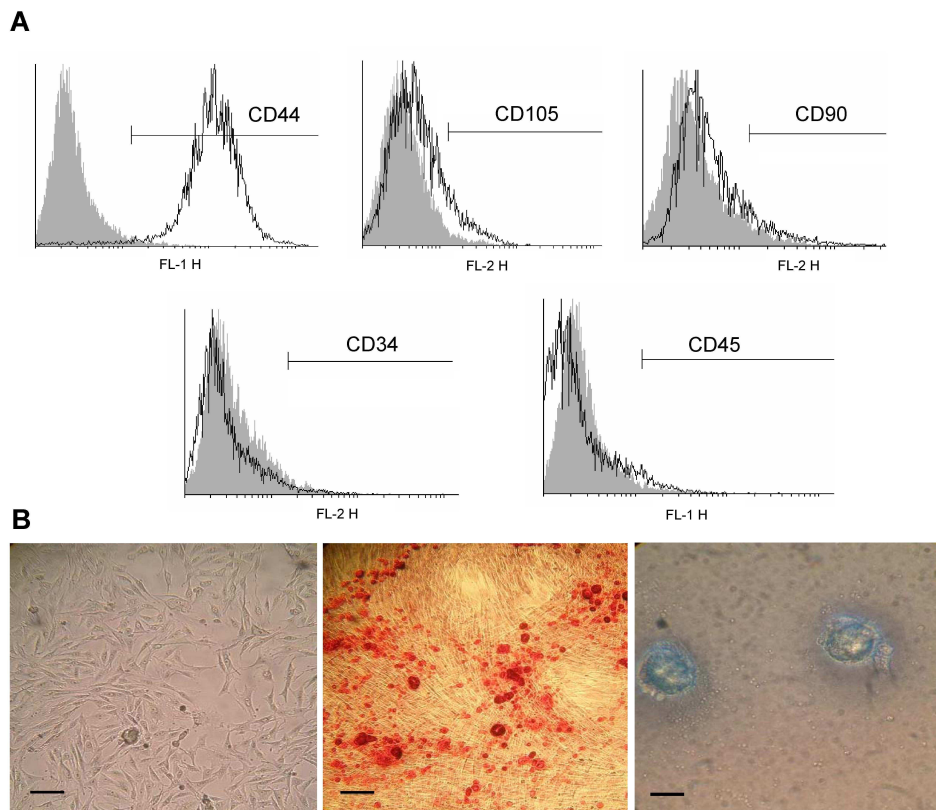
Data analysis was performed using one- or two-way ANOVA followed by Bonferroni tests. Results are expressed as mean \pm SD. Ischemic muscle damage was analyzed using a Fisher's exact test and expressed as percent samples displaying ischemic lesions. Statistical differences were considered significant at $p < 0.05$.

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Supplemental Figure I



Supplemental Figure I. A, Adipose stromal cells were positive for CD44, mildly positive for CD105 and CD90, and negative for CD34 and CD45. **B**, Cultured adipose stromal cells (left) were able to differentiate into osteogenic (mid) and chondrogenic (left) lineages. Bar: 50 μ m.

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Vascular Endothelial Growth Factor Overexpression Does Not Enhance Adipose Stromal Cell–Induced Protection on Muscle Damage in Critical Limb Ischemia

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