

Novel Cell-Free Strategy for Therapeutic Angiogenesis: In Vitro Generated Conditioned Medium Can Replace Progenitor Cell Transplantation

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Background

„Current evidence suggests that endothelial progenitor cells (EPC) contribute to ischemic **tissue repair by both secretion of paracrine factors and incorporation into developing vessels.** We tested the hypothesis that cell-free administration of paracrine factors secreted by cultured EPC may achieve an angiogenic effect equivalent to cell therapy.“

Conditioned medium preparation

Secretion of growth factors by EPC is increased by hypoxia

Table 1. Concentration of selected angiogenic growth factors in EPC-CM.

Cytokine/Growth factor	Concentration (pg/ml)	
	Hypoxia	Normoxia
IL-8/CXCL8	29090.7 ± 12279.4	2282.1 ± 406.3
SDF-1/CXCL12	6059.9 ± 654.6	3179.9 ± 488.0
HGF	539.5 ± 141.7	343.4 ± 74.8
Angiogenin	144.6 ± 68.2	72.5 ± 15.8
PDGF-BB	111.6 ± 27.02	19.9 ± 2.2
VEGF-A	25.5 ± 4.8	11.4 ± 5.2

Selected cytokine levels were measured in the conditioned media from culture expanded EPC incubated in hypoxic or normoxic condition for 72 hours.
doi:10.1371/journal.pone.0005643.t001

Cell survival assay

EPC-CM enhances endothelial cell-viability *in vitro*

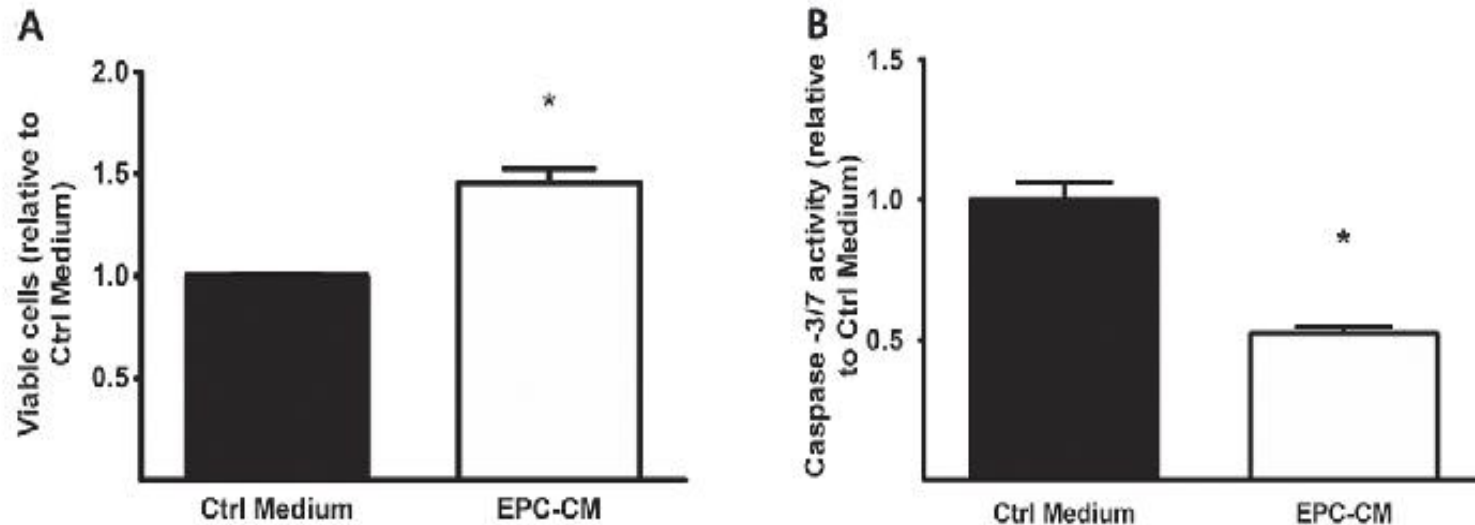


Figure 1. Pro-survival properties of EPC-CM. Serum starved HUVEC were incubated in EPC-CM or control medium for 24 hrs and analyzed for cell survival and extent of apoptosis. **(A)** The number of viable cells was assessed by CyQuant[®] NF and expressed relative to control. **(B)** Apoptosis was measured by the level of caspase -3/7 activity by Apo-ONE[®] and expressed relative to control. *, $P < 0.001$.
doi:10.1371/journal.pone.0005643.g001

In vitro angiogenesis assay

EPC-CM increases vascular sprouting

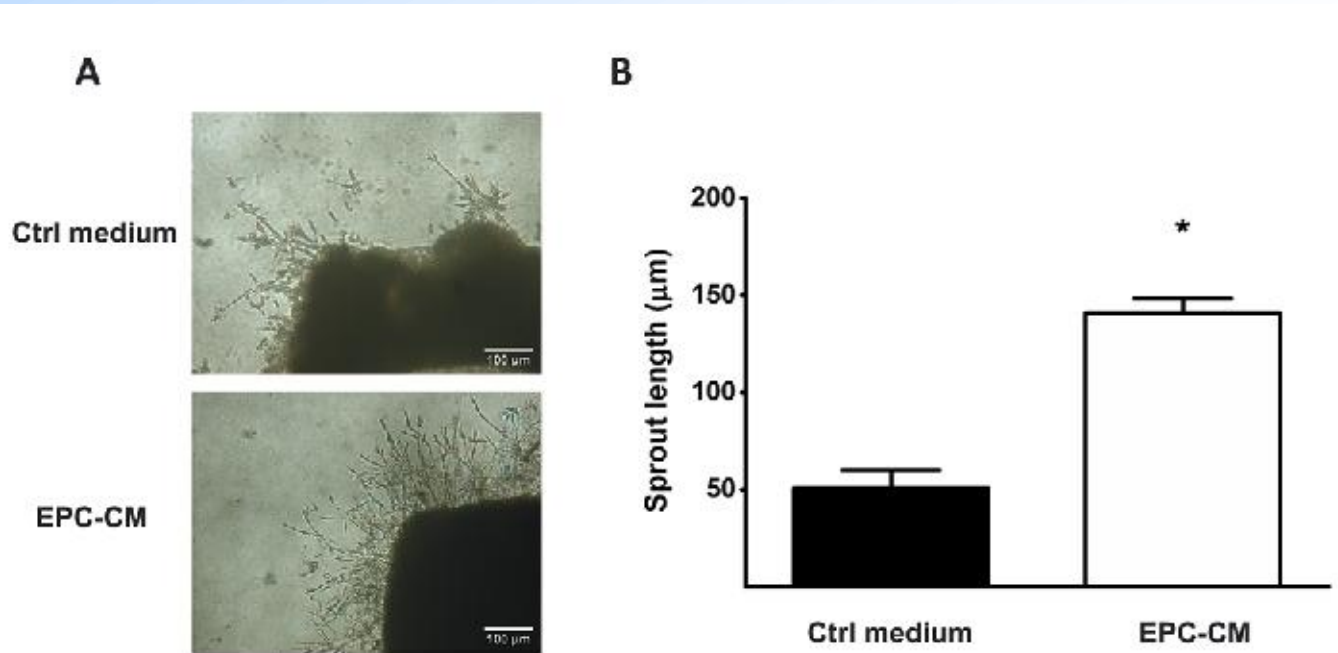
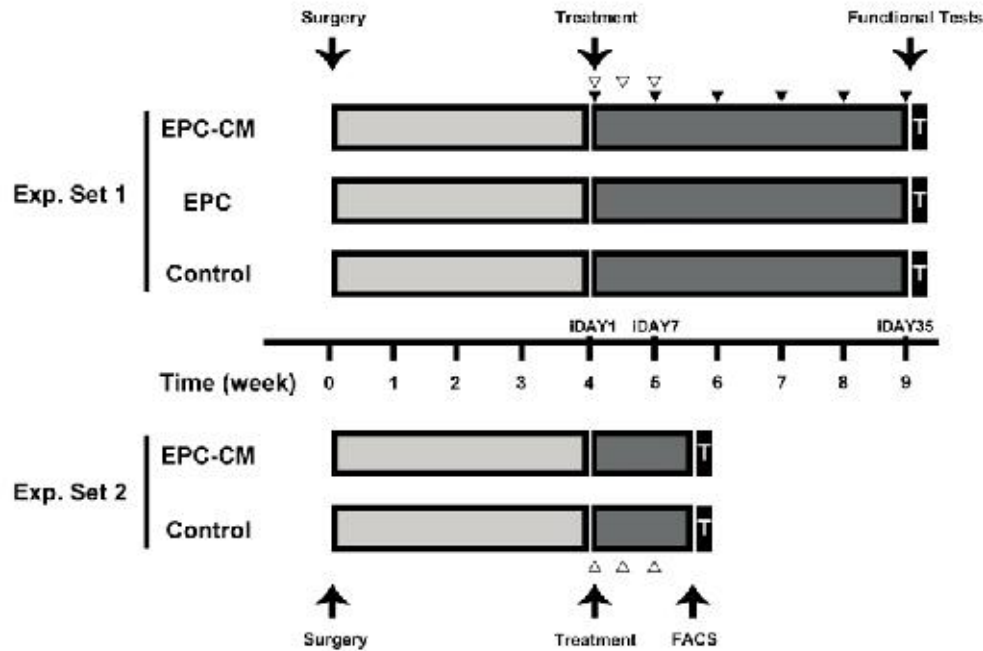


Figure 2. Angiogenic potential of EPC-CM. (A) Representative pictures of vascular outgrowth from 1 mm rat aortic ring embedded in growth factor reduced-MatrigelTM and incubated with EPC-CM or control medium. Incubation with EPC-CM enhanced the formation of capillary outgrowth compared to control medium. (B) Quantitative analysis of sprout length induced by incubation with control medium and EPC-CM. *, $P < 0.001$. doi:10.1371/journal.pone.0005643.g002

Design of *in vivo* experiments



Long term effect on hindlimb perfusion and function

Stimulation and recruitment of host cells

Figure 3. Design of *in vivo* experiments. Two *in vivo* experimental settings were designed to address the effect of the treatment modalities on tissue regeneration and neovascularization (Exp. Set 1) as well as progenitor cells mobilization and recruitment (Exp. Set 2). In both settings, rats were treated by 3 separate intramuscular injections within 7 days (iDAY1- iDAY7), 4 weeks after inducing ischemia as indicated by the white arrowheads (▽). Black arrowheads (▼) indicate blood flow measurements by Laser-Doppler of the hindlimb. T indicates tissue harvest and immunohistochemistry analysis. doi:10.1371/journal.pone.0005643.g003

Laser Doppler blood perfusion imaging

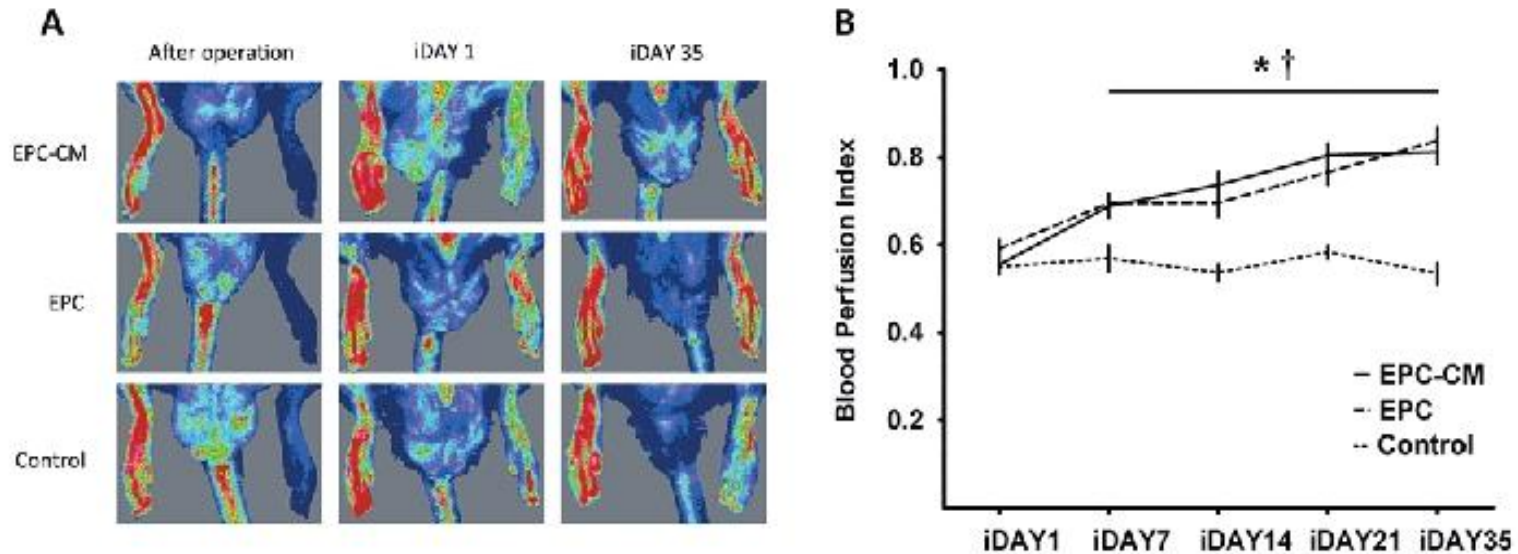


Figure 4. EPC-CM and EPC transplantation improve blood perfusion in the ischemic hindlimb. (A) Representative images of hindlimb blood flow measured by laser Doppler immediately after intramuscular injection of EPC-CM, EPC or control medium (iDAY1, 4 weeks after occlusion of the femoral artery) and the end of the experiment (5 weeks after treatment, iDAY35). (B) Quantitative analysis of blood flow expressed as perfusion ratio of the ischemic to the contralateral (non-operated) hindlimb over the observation period (iDAY1: day of EPC-CM or EPC injection; iDAY7; iDAY14; iDAY21; iDAY28 and iDAY35: 1, 2, 3, 4 and 5 weeks after injection, respectively). *, EPC-CM vs. Control, $P < 0.01$; †, EPC vs. Control, $P < 0.01$. doi:10.1371/journal.pone.0005643.g004

Assesment of hindlimb

Forced swimming test and mitochondrial acitivity

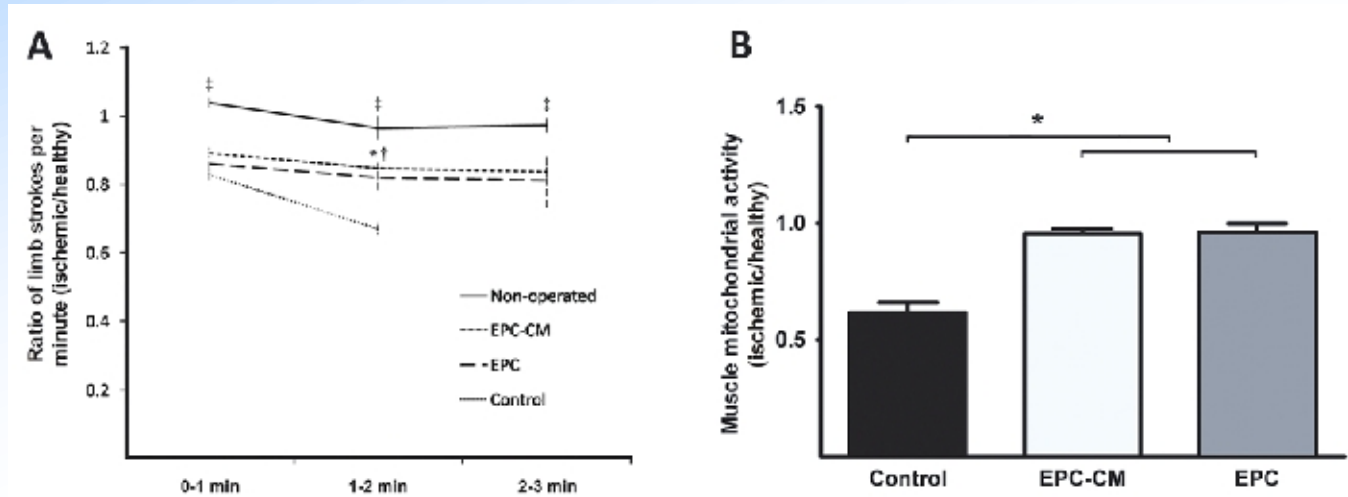
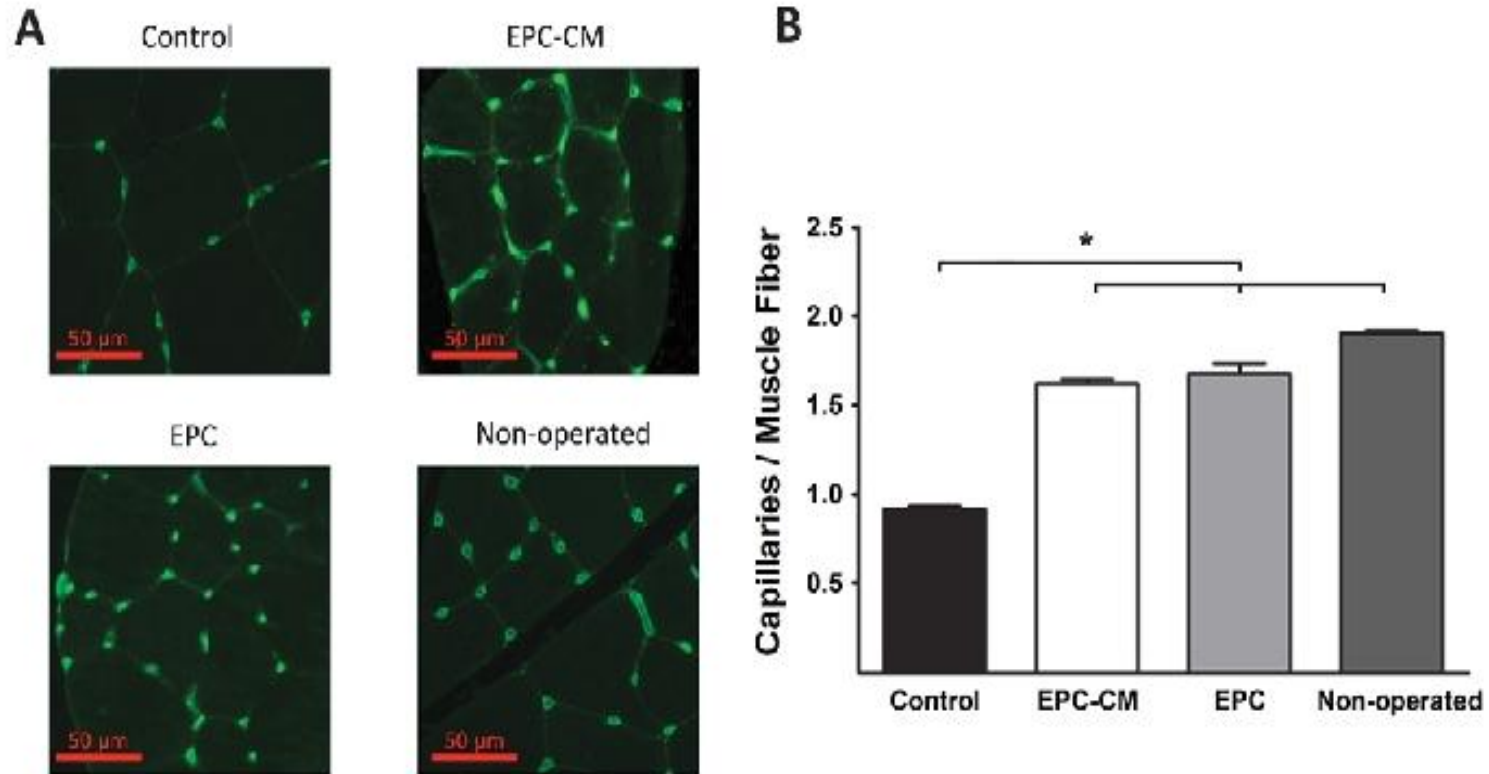


Figure 5. Effect of EPC-CM and EPC transplantation on ischemic muscle function and activity. (A) Muscle function was tested by swimming exercise and expressed as the ratio of ischemic to healthy hindlimb stroke numbers in animals treated with EPC-CM, EPC, control medium or non-operated animals. Swimming activity was monitored for 3 minutes at 1 minute intervals. Rats treated with control medium were not able to complete the exercise due to obvious exhaustion with drowning. *, EPC-CM vs. Control, $P < 0.05$; †, EPC vs. Control, $P < 0.05$; ‡ Non-operated vs. EPC-CM and EPC, $P < 0.05$. **(B)** Muscle mitochondrial activity in animals treated with EPC-CM, EPC or control medium was assessed by MTT reduction in the healthy and ischemic hindlimbs. The activity index is indicated as the ratio ischemic to healthy MTT values per gram of dry tissue. *, $P < 0.05$. doi:10.1371/journal.pone.0005643.g005

Capillaries stained with BS-1 lectin



Stained NG2+ pericytes & vWF

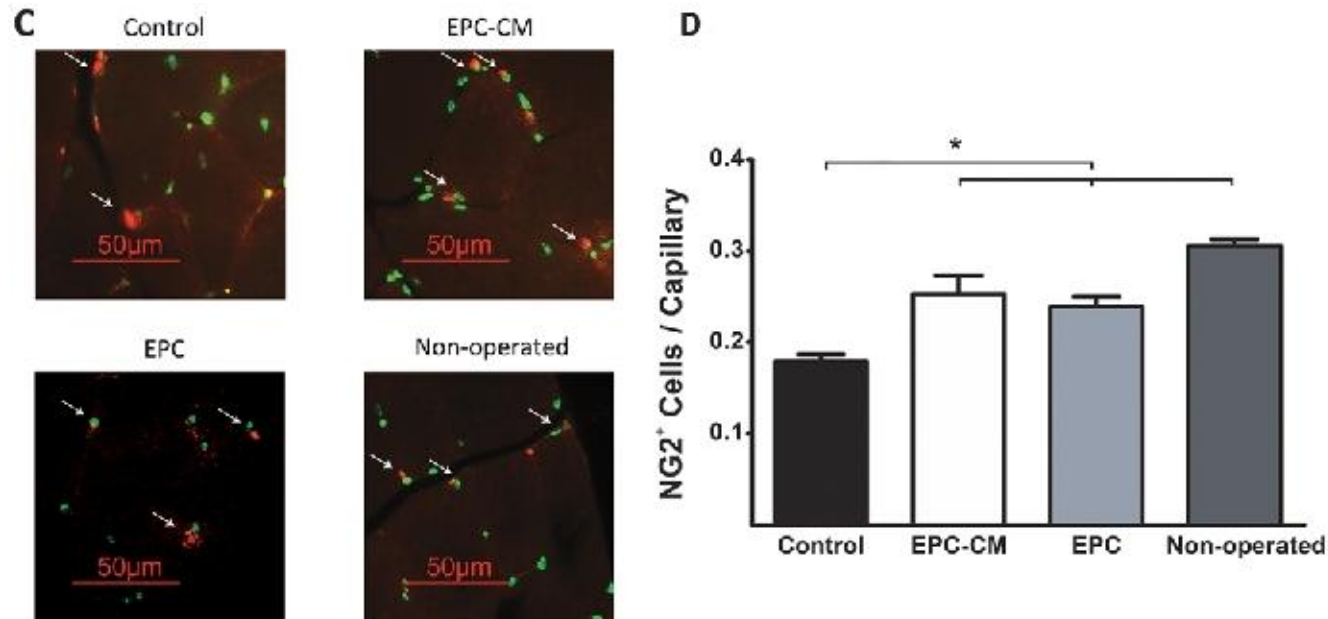


Figure 6. Effect of EPC-CM and EPC transplantation on ischemic muscle neovascularization. (A) Representative images of healthy (non-operated) and ischemic hindlimb muscle of animals treated with EPC-CM, EPC or control medium stained with BS-1 lectin (FITC) to localize capillaries. (B) Quantitative analysis of capillary density expressed by the number of capillaries per muscle fiber. *, $P < 0.05$. (C) NG2⁺ pericytes (white arrows) were identified (red fluorescence) by being adjacent to endothelial cells stained for von Willebrand Factor (green fluorescence). (D) Quantitative analysis of NG2⁺ cells per capillary in healthy and ischemic hindlimbs treated with EPC-CM, EPC and control medium. *, $P < 0.05$. doi:10.1371/journal.pone.0005643.g006

Stained alpha-smooth muscle actin

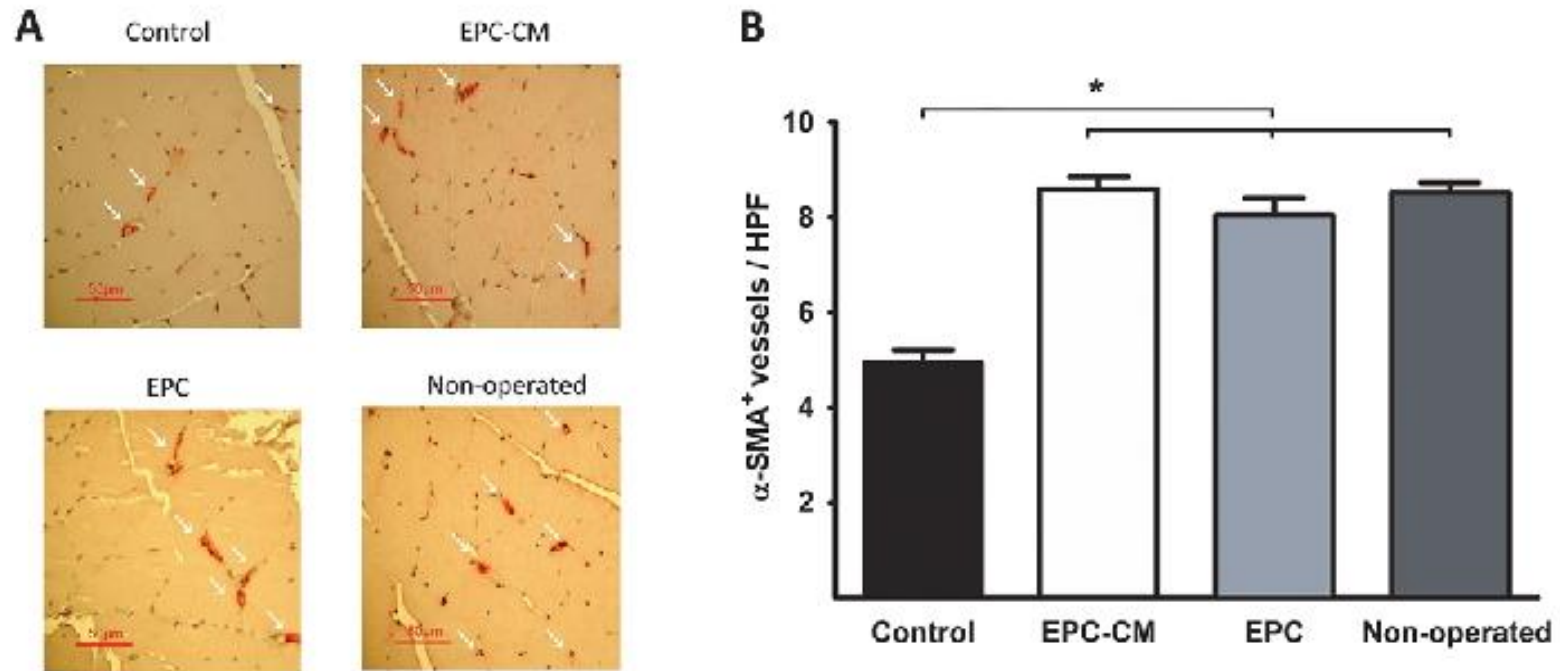
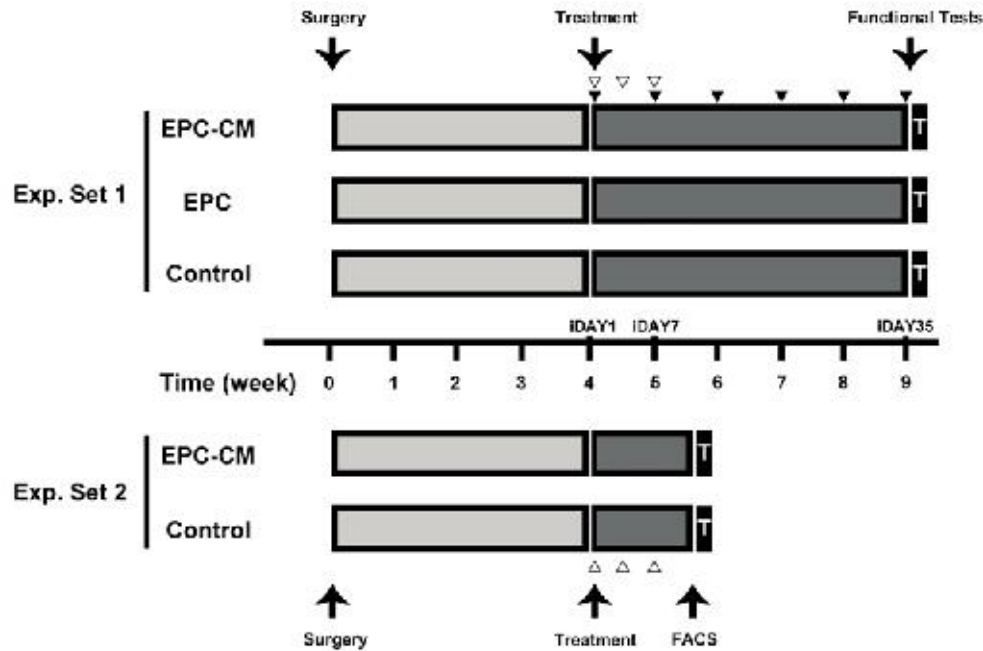


Figure 7. Effect of EPC-CM and EPC transplantation on vascular maturation. (A) Representative images of healthy and ischemic hindlimb muscle of animals treated with EPC-CM, EPC or control medium stained with α -smooth muscle actin (α -SMA) to evidence vascular maturation (red staining, white arrows). (B) Quantitative analysis of α -SMA⁺ vessels per high power field (HPF). *, $P < 0.05$.
doi:10.1371/journal.pone.0005643.g007

Design of *in vivo* experiments



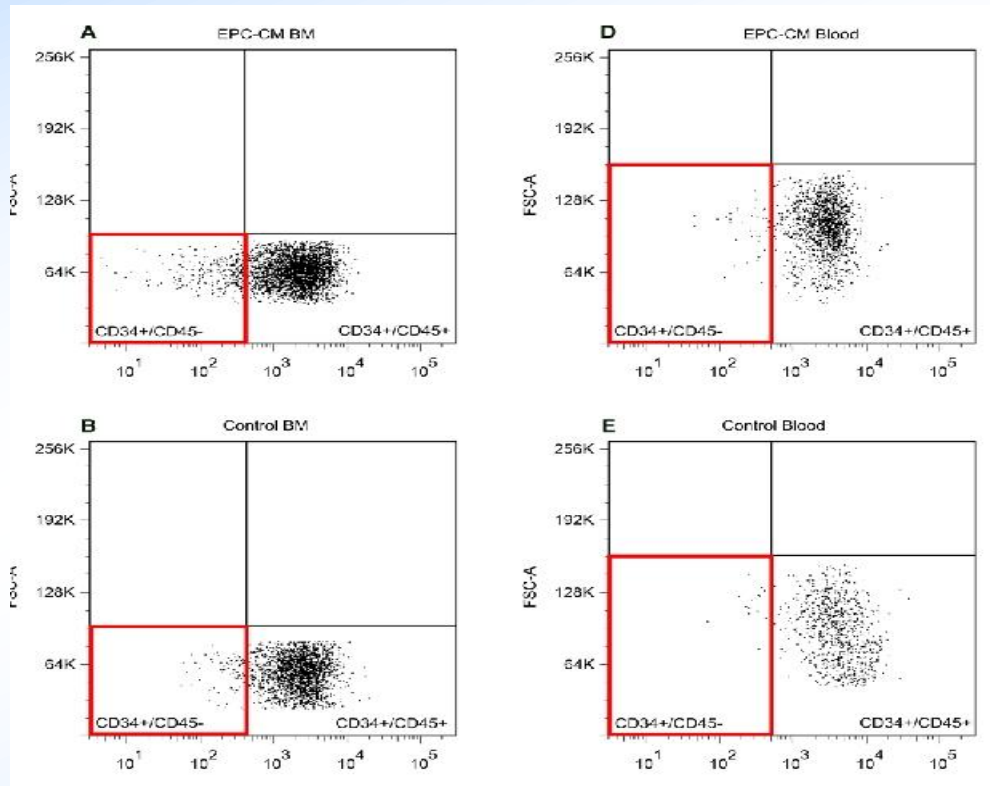
Long term effect on hindlimb perfusion and function

Stimulation and recruitment of host cells

Figure 3. Design of *in vivo* experiments. Two *in vivo* experimental settings were designed to address the effect of the treatment modalities on tissue regeneration and neovascularization (Exp. Set 1) as well as progenitor cells mobilization and recruitment (Exp. Set 2). In both settings, rats were treated by 3 separate intramuscular injections within 7 days (iDAY1- iDAY7), 4 weeks after inducing ischemia as indicated by the white arrowheads (▽). Black arrowheads (▼) indicate blood flow measurements by Laser-Doppler of the hindlimb. T indicates tissue harvest and immunohistochemistry analysis. doi:10.1371/journal.pone.0005643.g003

Progenitor cells mobilization

EPC-CM stimulates the mobilization of bone-marrow derived EPC



Quantitative analysis

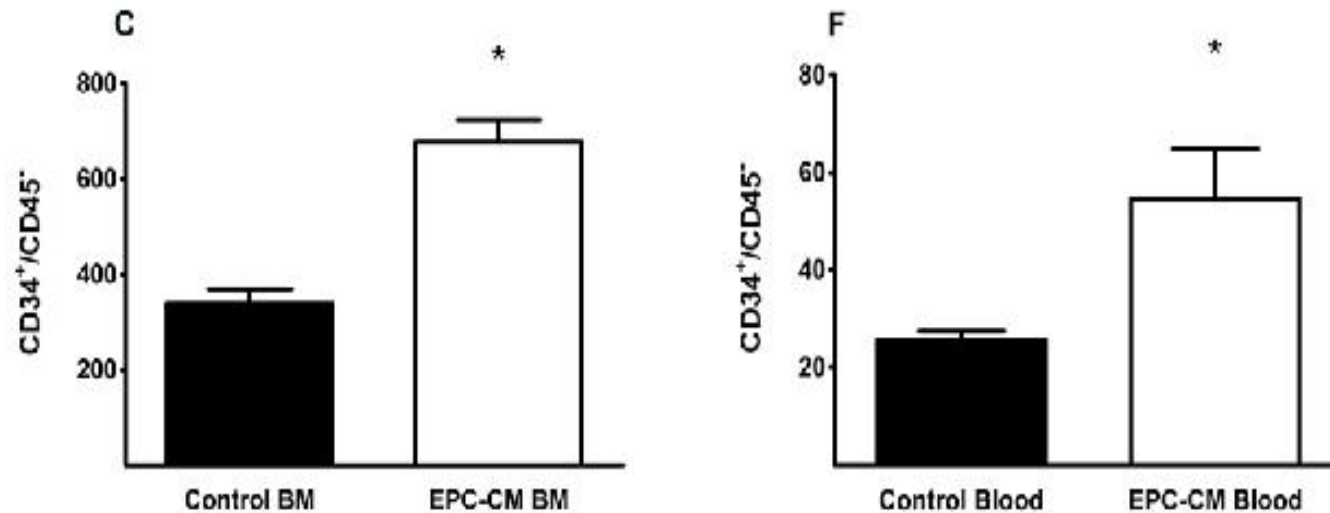


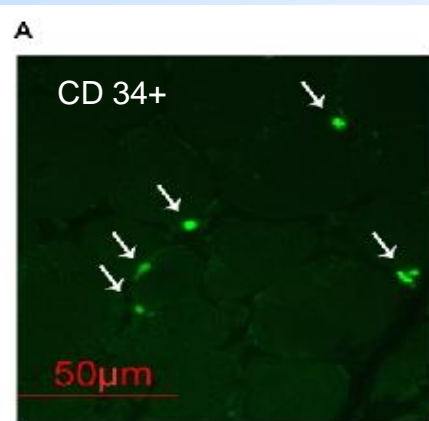
Figure 8. EPC-CM stimulates the mobilization of bone marrow-derived EPC. Representative FACS analysis charts of CD34⁺/CD45⁻ cells isolated from bone marrow (A and B) and peripheral blood (D and E) of EPC-CM and control media treated animals 3 days after the last intramuscular injection. Quantitative analyses show significantly increased numbers of CD34⁺/CD45⁻ progenitor cells in the BM (C), and the peripheral blood (F) of EPC-CM treated animals. *, $P < 0.05$.

doi:10.1371/journal.pone.0005643.g008

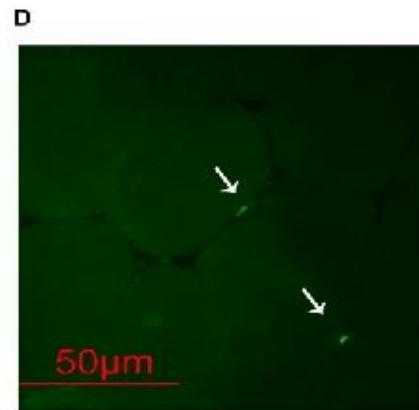
Immunofluorescence staining

EPC-CM promotes progenitor cells homing to the ischemic tissue

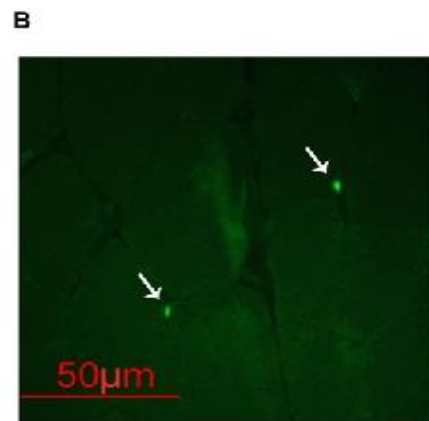
Day 10
EPC-CM treated



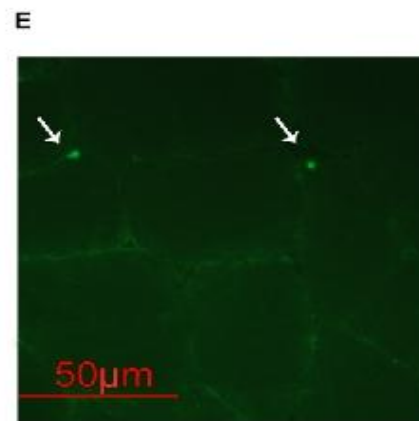
Day 35
EPC-CM treated



Day 10
Control treated



Day 35
Control treated



Temporary recruitment of CD34+ cells to the ischemic limbs to similar extent in different anatomic regions

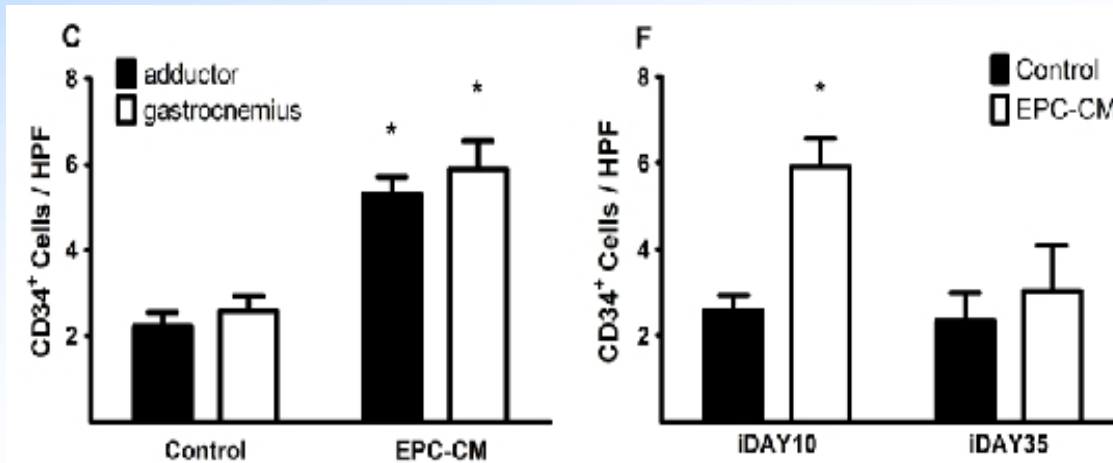


Figure 9. EPC-CM promotes progenitor cells homing to the ischemic tissue. Representative fluorescence pictures of CD34⁺ immunostaining in ischemic hindlimb tissue 3 days (left panel, iDAY10) and 4 weeks after treatment (right panel, iDAY35). The number of CD34⁺ cells on iDAY10 was significantly higher in EPC-CM treated limbs (A) as compared to control treated animals (B) with no evidence for focal recruitment, as CD34⁺ cells were found to similar extent in different anatomic regions (C). In comparison, 4 weeks after treatment (iDAY35), tissue sections from show decreased numbers of CD34⁺ cells in EPC-CM treated limbs (D) equivalent to numbers found in control (E). Quantitative analysis is depicted reflects the temporary recruitment of CD34⁺ cells to the ischemic limbs in EPC-CM treated animals (F). *, P<0.001.
doi:10.1371/journal.pone.0005643.g009

Discussion

Molecular effectors?

Interaction of downstream signals?

Conclusions

„Intramuscular injection of **EPC-CM** is as effective as **cell transplantation** for promoting tissue revascularization and functional recovery. Owing to the technical and practical limitations of cell therapy, cell free conditioned media may represent **a potent alternative for therapeutic angiogenesis in ischemic cardiovascular diseases.**“