α6-Integrin Subunit Plays a Major Role in the Proangiogenic Properties of Endothelial Progenitor Cells

Claire Bouvard, Benjamin Gafsou, Blandine Dizier, Isabelle Galy-Fauroux, Anna Lokajczyk, Catherine Boisson-Vidal, Anne-Marie Fischer, Dominique Helley

Objective—Alpha6 integrin subunit (α6) expression is increased by proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor. This increase correlates with enhanced in vitro tube formation by endothelial cells and their progenitors called Endothelial Colony-Forming Cells (ECFCs). We thus studied the role of α6 in vasculogenesis induced by human ECFCs, in a mouse model of hindlimb ischemia.

Methods and Results—We used small interfering RNA (siRNA) to inhibit α6 expression on the surface of ECFCs. For in vivo studies, human ECFCs were injected intravenously into a nude mouse model of unilateral hind limb ischemia. Transfection with siRNA α6 abrogated neovessel formation and reperfusion of the ischemic hind limb induced by ECFCs (P<0.01 and P<0.001, respectively). It also inhibited ECFC incorporation into the vasculature of the ischemic muscle (P<0.001). In vitro, siRNA α6 inhibited ECFC adhesion (P<0.01), pseudotube formation on Matrigel, migration, and AKT phosphorylation (P<0.0001), with no effect on cell proliferation or apoptosis.

Conclusion—α6 Expression is required for ECFC migration, adhesion, recruitment at the site of ischemia, and the promotion of the postischemic vascular repair. Thus, we have demonstrated a major role of α6 in the proangiogenic properties of ECFCs. (Arterioscler Thromb Vasc Biol. 2010;30:1569-1575.)

Key Words: adhesion molecules ■ angiogenesis ■ ischemia ■ peripheral arterial disease ■ vascular biology

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vitro. To our knowledge, the role of α6 expression by ECFCs in adult vasculogenesis has not been investigated. Therefore, we examined the influence of α6 expression on the proangiogenic properties of ECFCs and especially on its possible involvement in ECFC recruitment to sites of ischemia. We used small interfering RNA (siRNA) duplexes to inhibit α6 expression at the surface of human ECFCs and then examined the effect of this inhibition on adhesion, migration and pseudotube network formation in vitro, and revascularization in a nude mouse model of hind limb ischemia.

**Methods**

**ECFC Isolation and Culture and RNA Interference**

ECFCs were isolated, cultured, and characterized as previously described and as detailed in supplemental Figure I (available online at http://atvb.ahajournals.org). The ECFCs were transfected as described in the Supplemental Data.

**Mouse Model of Unilateral Hind Limb Ischemia**

All the protocols were approved by the Regional Ethics Committee on Animal Experimentation (ref P2.CBV.031.07), and all experiments conformed to European Community guidelines for the care and use of laboratory animals.

Male athymic nude Foxn-1 mice (Harlan, Gannat, France), aged 7 weeks and weighing 20 to 30 g, were anesthetized by isoflurane inhalation; the left femoral artery was ligatured. Five hours after the onset of ischemia, the mice received an intravenous injection of 100 µL of vehicle (PBS), 10^5 transfected ECFCs, or 10^5 untransfected ECFCs. Cells were transfected 96 hours before being injected into the animals.

After 2 weeks, mice were anesthetized with pentobarbital and placed on a heating pad at 37°C; laser Doppler perfusion imaging was used to assess leg tissue perfusion, as previously described. The ECFCs were transfected as described in the Supplemental Data.

**Capillary Density Determination**

Frozen 10-µm-thick sections of the distal part of the gastrocnemius muscle were fixed in ice-cold acetone for 10 minutes and incubated for 1 hour with a rat anti–mouse CD31 monoclonal antibody (clone J70A; DAKO, Glostrup, Denmark), and nuclei were stained with TOPRO3 iodide. The mouse vasculature was stained with a rat anti–mouse CD31 monoclonal antibody. Nuclei were stained with TOPRO3 iodide. Incorporated ECFCs were detected with a confocal microscope, and the results were expressed as the number of incorporated ECFCs per section.

**In Vitro Assay 72 Hours After Transfection of ECFCs**

**Adhesion Assay**

Plates (with 96 wells) were coated with Matrigel, and nonspecific binding sites were saturated with BSA in PBS for 1 hour at room temperature. The wells were then washed 3 times with adhesion buffer (10-mmol/L Hepes; 140-mmol/L NaCl; 5.56 mmol/L glucose; 1% BSA; 5.4 mmol/L potassium chloride; 2 mmol/L calcium chloride; and 1 mmol/L magnesium chloride pH 7.4) and then ECFCs suspended in adhesion buffer were distributed (30 000 cells per well). After 20 minutes at 37°C with 5% CO2, nonadherent cells were removed and the wells were washed 3 times. The number of adherent cells was determined by a p-nitrophenyl phosphate colorimetric assay. The results were normalized to the untransfected ECFC group.

**Migration Assay**

For the Boyden chamber migration assay, cell culture inserts (8 µm pore size) were placed in 24-well plates and coated with laminin. Endothelial basal medium (EBM)-2–containing 5% FCS, with or without 40-ng/mL VEGF, was placed in the lower compartment of the Boyden chamber. ECFCs suspended in EBM-2 containing 5% FCS were placed in the upper compartment (15 000 cells per well). After 6 hours at 37°C with 5% CO2, cells adhering to the lower surface of the inserts were counted in 10 different fields per well using a microscope with a grid eyepiece. Results are expressed as the difference between experiments with and without VEGF and are normalized to the untransfected ECFC group.

**Matrigel Tube Formation Assay**

ECFCs suspended in EBM-2 containing 5% FCS were distributed (30 000 per well) on 48-well plates coated with growth factor–reduced Matrigel. They were allowed to form pseudotubes for 18 hours at 37°C with 5% CO2. The total length of the pseudotubes was quantified with computer software (Videomet; Microvision Instruments, Evry, France). The results are expressed as the ratio of the ischemic (left) leg to the nonischemic (right) leg.

**Statistical Analysis**

Results are expressed as mean±SEM of at least 3 experiments. Data were analyzed by ANOVA, followed by the Fisher protected least
A single intravenous injection of control ECFCs (n=8) increased the ischemic to nonischemic leg blood flow ratio relative to PBS-treated mice by 60% (P<0.001). ECFCs transfected with scramble siRNA (n=10) also enhanced the perfusion in the ischemic hind limb, with no significant difference from untransfected ECFCs. In contrast, ECFCs transfected with siRNA a6 (n=11) did not improve reperfusion, with an ischemic to nonischemic leg blood flow ratio as low as PBS-injected mice (n=8). Thus, the inhibition of a6 expression significantly inhibited the ability of ECFCs to promote the reperfusion of the ischemic hind limb (P<0.001 versus untransfected ECFCs and P<0.01 versus ECFCs transfected with scrambled siRNA) (Figure 1).

Capillary Density
A single injection of untransfected ECFCs (n=6) or ECFCs transfected with scramble siRNA (n=10) increased capillary density in the ischemic gastrocnemius muscle by a factor of 2 relative to PBS-injected mice (P<0.01). Once again, for the group injected with ECFCs transfected with siRNA a6 (n=12), the ratio of ischemic to nonischemic leg capillary density was as low as PBS-injected mice (n=8). The inhibition of a6 expression by the siRNA cancelled the beneficial effects of ECFCs on capillary density (P<0.01 versus untransfected ECFCs, and P<0.001 versus ECFCs transfected with scramble siRNA) (Figure 2).

Histomorphometric Analysis
The left gastrocnemius was more severely necrotic in animals injected with PBS (n=4) or with ECFCs transfected with siRNA a6 (n=6) than in animals injected with untransfected ECFCs (n=4) or ECFCs transfected with scramble siRNA (n=5). Quantitative analysis showed that the injection of ECFCs transfected with scramble siRNA and untransfected ECFCs reduced the percentage of necrotic tissue by a factor of 2 relative to the PBS-treated animals (P<0.05 and P<0.01, respectively). In contrast, ECFCs transfected with siRNA a6 had no beneficial effect relative to PBS-treated controls (P>0.001 versus untransfected ECFCs, and P<0.01 versus ECFCs transfected with scramble siRNA) (Figure 3). These results are in accordance with TUNEL analysis (supplemental Figure IV).

Loss of a6 Reduces ECFC Incorporation Into the Microvasculature of Ischemic Skeletal Muscle
We evaluated the number of human ECFCs (labeled red with an anti–human CD31 antibody) incorporated into the mouse microvasculature (labeled green). ECFCs were found in the ischemic leg but not in the healthy leg. siRNA a6–transfected ECFCs were incorporated 5 times less efficiently than un-
Transfection with scramble siRNA or siRNA

Apoptosis or Viability

5), or ECFCs transfected with scramble siRNA (n=5 per group, P<0.0001 for all) (Figure 6).

Discussion

This study demonstrates the importance of α6 in the proangiogenic properties of ECFCs. As previously reported, ECFCs injected intravenously into a nude mouse model of hind limb ischemia improved neovessel formation and reperfusion\textsuperscript{15–17} and provided protection toward necrosis. However, inhibition of ECFC cell surface α6 expression by using specific siRNA abrogated all these beneficial effects.

To understand why the cells lacking α6 were unable to improve neovessel formation and reperfusion, we investigated the role of α6 in the homing of ECFCs, which is a key step in cell therapy. Indeed, some studies showed that soon after the injection, the major part of the cells is removed from the blood circulation and found mainly in the spleen, liver, and kidneys. Despite this loss, the remaining cells are located in the ischemic area.\textsuperscript{18} ECFCs that have been attracted there by VEGF or stromal cell derived factor (SDF)-1 can incorporate the damaged vasculature and form new blood vessels, unlike colony-forming unit endothelial cells, which promote angiogenesis only through the release of proangiogenic factors and cytokines.\textsuperscript{11} Even if it is still unclear, ECFCs may also secrete proangiogenic factors, such as placental growth factor-1 (PIGF)\textsuperscript{17} and prostaglandin,\textsuperscript{16} which could explain why ECFC injection can increase neovessel formation even with few cells found in the ischemic area.

Qian et al\textsuperscript{8} showed that α6 is involved in the homing of hematopoietic stem cells to bone marrow in a model of cell transplantation in irradiated mice. These researchers suggested that α6 contributes to hematopoietic stem cell transmigration to bone marrow because it serves as a receptor for ECM laminins, which are involved in regulating tissue organization, cell adhesion, differentiation, and migration.\textsuperscript{19} Other researchers reported that a subpopulation of mesenchymal stem cells, expressing high levels of α6, showed increased migration to infarcted heart in mice.\textsuperscript{20} To determine whether α6 is involved in the homing of ECFCs, we quantified the number of ECFCs incorporated into the vasculature of skeletal muscles 4 days after their injection, as described by Foubert et al.\textsuperscript{15} When cell surface α6 expression was inhibited by siRNA, the number of ECFCs integrated into the mouse microvasculature of the ischemic muscle was reduced 5-fold. To understand why the cells lacking α6 were not recruited and integrated to the ischemic mouse vasculature, we performed in vitro assays.

After the obstruction of an artery, the oxygen supply is reduced and, therefore, the endothelial cells lining the walls of the downstream vessels undergo hypoxia. The death of these endothelial cells leaves the basement membrane partially uncovered. The integrin α6β1 is a receptor for laminin, the main component of the basement membrane. Consequently, α6 could be implicated in ECFC adhesion to the basement membrane of the injured blood vessels located in the ischemic area (supplemental Figure VI and supplemental Figure VII). Although other integrins or adhesion proteins are
also involved, when cell surface α6 expression was inhibited by siRNA, ECFC adhesion to Matrigel, a substitute of the basement membrane, was reduced by a factor of 2. Moreover, we verified that siRNA α6 had no effect on ECFC proliferation, apoptosis, or viability. These results suggest that the low number of ECFCs transfected with siRNA α6 found in the ischemic muscles was the result of poor attachment and was not a bias due to decreased cell proliferation or viability.

Once endothelial progenitors have adhered, they must migrate to participate to the remodeling and to form new blood vessels. At sites of ischemia, VEGF and other angiogenic factors act as chemoattractants for cells involved in neovascularization. By using a Boyden chamber migration assay, we found that a lack of α6 expression inhibited ECFC migration induced by VEGF. These observations are in keeping with results previously obtained with other cell types. On human brain microvascular endothelial cells, α6β1 is involved in VEGF-induced adhesion, migration, and in vitro angiogenesis. α6 Overexpression on hepatocarcinoma cells leads them to acquire an invasive phenotype. Integrin α6β1 is necessary for matrix-dependent focal adhesion kinase (FAK) activation and, therefore, for the migration of hepatocarcinoma cells. It is also involved in the attachment of these cells to laminin. The same phenomenon has been observed in breast cancer, where α6 promotes carcinoma survival and progression. By using a different approach, our findings support the hypothesis that α6 could be involved in the mobilization and migration of the progenitors or stem cells from their niches.
When ECFCs are recruited to sites of ischemia, they can participate in vascular repair by either exerting paracrine effects or directly forming new blood vessels. Previous experiments with anti-α6 antibodies have shown that α6 is involved in endothelial cell cord formation in vitro. Thus, we used the Matrigel model to examine the role of α6 in vessel formation. We found that ECFC transfection with siRNA α6 strongly inhibited vascular network formation, suggesting a crucial role of α6 in ECM-mediated migration and differentiation and, consequently, in new blood vessel sprouting, orientation, and stabilization during angiogenesis. Also, siRNA α6 inhibits cordlike network formation by human breast cancer cells. α6 Is involved in ECFC adhesion, migration, and pseudo-tube formation. To understand why, we investigated different signaling pathways (AKT, extracellular signal regulated kinase, and p38), and we observed that AKT phosphorylation was reduced by a factor of 2, 1 hour after adhesion on laminin, when α6 was knocked down. This result could explain the observed cellular effects because the phosphatidylinositol 3-kinase/AKT pathway has been shown to be implicated in endothelial progenitor migration and adhesion.

However, ECFCs may also promote angiogenesis via indirect effects, such as interactions with other cell types. Interestingly, α6 can mediate cell-cell interactions independently of laminin. For example, α6β1 has a key role in gamete fusion, resulting from an interaction with membrane-anchored cell surface ligands from the A Disintegrin and Metalloproteinase (ADAM) family. Interaction with ADAM-9 is also responsible for the induction of fibroblast motility. The role of α6 in ECFC interaction with other cell types should be further investigated.

In conclusion, α6 plays a major role in the proangiogenic properties of ECFCs. α6 Is involved in ECFC adhesion to the basement membrane and in migration toward VEGF, explaining why this integrin subunit is required for ECFC recruitment to the site of ischemia and for the formation of vascular tube networks. A better understanding of the phenomenon involved in cell recruitment at the site of injury could allow us to find new strategies to enhance cell therapy efficiency. Regarding human cell therapy, our results suggest that enhancing α6 expression on ECFCs might improve their recruitment to sites of ischemia and stimulation.
promote vascular repair.\textsuperscript{33} On the other hand, ECFCs are involved in tumor angiogenesis,\textsuperscript{34} and \(\alpha 6\) plays a role in both tumor angiogenesis and growth.\textsuperscript{35} Therefore, our findings support the possibility that \(\alpha 6\), like other integrins, might be an interesting therapeutic target for strategies designed to disrupt tumor angiogenesis.\textsuperscript{35}

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Disclosures

None.

References

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SUPPLEMENT MATERIAL

Detailed Methods:

ECFC isolation and culture
Mononuclear cells were isolated from human umbilical cord blood by density gradient centrifugation as previously described. After an adhesion step, CD34+ cells were selected by magnetic activated cell sorting. The cells thus collected were plated on 0.2% gelatin-coated 24-well plastic culture dishes at a density of 5x10^5 cells/well in EGM-2 medium (Lonza, Walkersville, MD, USA). After 4 days, non adherent cells were removed and the medium was renewed. After 10 days of culture, ECFC colonies became visible microscopically. Cells were then detached with trypsine and expanded in EGM-2 on 0.2% gelatin coated plates and grown at 37°C in a humidified 5% CO2 atmosphere for further analysis. ECFC were used 25 to 45 days after cord blood processing. Characterization of ECFCs is described below.

Flow cytometric characterization of ECFCs
ECFCs were detached with accutase (PAA, Laboratory, Linz, Austria), washed in HBSS containing 2% FCS, resuspended at 5x10^6 cells/mL, and incubated with the following antibodies CD31-FITC, CD14-FITC (Becton Dickinson, Franklin Lakes, NJ, USA), CD34-FITC, CD144-PE, CD146-PE, CD45-FITC, (Beckman Coulter, Fullerton, CA, USA) or an isotype control antibody from the same manufacturer. Ten thousand cells were analyzed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).
RNA interference

ECFCs were transfected at subconfluence by using the DharmaFECT-1 transfection reagent (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA) with pooled specific human α6 integrin subunit siRNA (Table S1) or with a non targeting control siRNA, following the reagent manufacturer’s protocol. Briefly, 24 hours before transfection, the medium was replaced by EGM-2 without antibiotics. For transfection, the siRNA solution was mixed with the transfection reagent in EBM-2 for 20 minutes and then added to antibiotic-free EGM-2. The cell culture medium was then replaced by this mix containing 60 nM of siRNA (Table I). The medium was renewed with EBM-2/5% FCS after 24 hours and then every 48 hours. Between 72 and 96 hours after transfection, cells were detached with accutase and used in the different assays, (always 96 hours after transfection for in vivo studies). Kinetics of α6 knockdown is described in supplemental figure II.

Gene expression analysis by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from ECFCs by using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) as recommended by the manufacturer. First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using the High Capacity cDNA Archive kit, random primer and Superscript II reverse transcriptase (Life Technologies, Foster City, CA, USA). Alpha6 gene expression was analyzed by qRT-PCR using an ABI Prism 7900 HT Sequence Detection System (Life Technologies, Foster City, CA, USA) with SYBR green reporter dye (Life Technologies, Foster City, CA, USA). The following primers were used: α6 sense, 5'-CACATCTCCTCCCTGACACA-3’; α6 antisense, 5’-TATATCTTGCACCCCATCCTT-3’; TBP sense, 5’-TGACACAGGAGCCAGAGTGA-3’; TBP antisense, 5’-TATATCTTGCACCCCATCCTT-5’. TBP, encoding the TATA box binding protein, was
used as the endogenous RNA control, and each sample was normalized on the basis of its TBP content. Results were normalized to non transfected ECFC control values.

**Flow cytometric analysis of α6 cell-surface expression**

At various culture times, cells were detached with Accutase (PAA Laboratory, Linz, Austria), washed in HBSS containing 2% FCS, resuspended at $5 \times 10^6$ cells/mL, and incubated with a PE-conjugated antibody against α6 (CD49f, clone G0H3, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) or an isotype-matched mouse irrelevant antibody from the same manufacturer. Five thousand cells were analyzed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). Results were normalized to non transfected ECFC control values.

**Flow cytometric analysis of CD31, CD144, α1, α2, α3, α4, α5, β1, β4, ανβ3 cell surface expression after transfection**

After 96 hours of transfection with siRNA, cells were detached with Accutase (PAA Laboratory, Linz, Austria), washed in HBSS containing 2% FCS, resuspended at $5 \times 10^6$ cells/mL, and incubated with the following antibodies CD31-FITC, CD49a-PE, CD49b-FITC, CD49e-PE, CD49e-PE, CD29-FITC, CD104-PE, CD1261/CD61-FITC (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), CD144-PE (Beckman Coulter, Fullerton, CA, USA) or an isotype-matched mouse irrelevant antibody from the same manufacturer. Ten thousand cells were analyzed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).
**In situ** apoptosis detection on ischemic muscles sections (TUNEL assay)

Frozen tissue sections (10µm) were dried, fixed in formaldehyde solution and labeled using TACS™ 2 TdT-DAB* In Situ* Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer’s protocol.

**Evaluation of Caspase 3 activity**

After 96 hours of transfection with siRNA, wells were washed and the medium was changed by a serum free medium. After six hours of serum starvation, cells were washed and lysed. Lysates were then used in the Caspase 3 colorimetric assay kit (assay designs, Ann Harbor, MI, USA), following the manufacturer’s protocol. The assay was done three times in duplicate. The results were normalized to the untransfected ECFC group and represented as means + SEM of three experiments.

**Evaluation of cell viability**

After 96 hours of transfection with siRNA, dead cells present in the supernatant were collected, and living adherent cells were detached and collected. After centrifugation, the pellets were resuspended, and cell suspension was diluted in trypan blue. The number of living cells (no coloration) and dead cells (blue coloration) present in each well was quantified using a Malassez cell and a microscope.

**Laminin, human CD31 and mouse CD31 labeling on ischemic and non ischemic muscles**

Frozen tissue sections (10µm) were fixed in ice-cold acetone, immunostained with a biotinylated anti-human CD31 antibody (clone JC70A, DAKO, Glostrup, Denmark), and incubated with streptavidin-Alexa 555 (Invitrogen, Carlsbad, CA, USA). The mouse vasculature was stained with a rat anti-mouse CD31 monoclonal antibody. And murine
laminin present in the muscles was stained with a rabbit anti-mouse laminin antibody. Nuclei were stained with TOPRO3 iodide. Sections were observed under a confocal microscope.
Figure I: Flow cytometric analysis of surface antigens on ECFCs.

ECFCs were positive for CD31, CD34, CD144 and CD146 but not for monocytic markers CD45 and CD14. Isotypic control is represented in grey. The microphotograph represents ECFCs, with their characteristic cobblestone shape.
Figure II: Assessment of siRNA efficiency by qRT-PCR (A) and flow cytometry (B): kinetics of cell-surface α6 expression and mRNA quantification.

ECFCs were cultured and transfected as described in Materials and Methods. At various times after this transfection, the quantity of α6 integrin subunit mRNA was determined by RT-PCR (A) and its protein expression at the ECFC surface was measured by flow cytometry (B).
Results for ECFCs transfected with DharmaFECT alone (□), scramble siRNA (☑), or siRNA α6 (☐) were normalized to untransfected control ECFCs (■). Results are means ± S.E.M. of three different experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Neither the transfection reagent nor the scramble siRNA degraded α6 mRNA or affected α6 expression at the cell surface (no significant difference with untransfected ECFCs). The siRNA directed against α6 efficiently induced α6 mRNA degradation for at least 144 hours (Figure S2A). As a result, α6 integrin subunit expression was reduced on ECFCs transfected with siRNA α6 (about 10% of control value from 48 h to 96 h (P<0.0001) and 20% after 120 h) (Figure S2B). We thus decided to use cells 72 hours after transfection for in vitro experiments and 96 hours after transfection for in vivo experiments.
Figure III: siRNA α6 has no effect on CD31, CD144, α1, α2, α3, α4, α5, β1, β4, ανβ3 cell surface expression after 96 hours of transfection.

These results show that siRNA α6 are specific and that the observed effects are due to the loss of α6 and not to off-targets.
Figure IV: *In situ* apoptosis detection on ischemic muscles sections (TUNEL assay)

Gastrocnemius sections (10µm) were stained using TACS™ 2 TdT-DAB *In Situ* Apoptosis Detection Kit. Apoptotic cells (dark brown nucleus), and necrotic cells (brown staining in the cytoplasm of enlarged cells) were more numerous in groups injected with PBS or with ECFC transfected with siRNA α6 than in groups injected with untransfected ECFCs or ECFCs transfected with scramble siRNA. Injection of ECFCs or ECFCs transfected with scramble siRNA protected tissues from apoptosis, but when α6 is knocked down, this beneficial effect is lost.
Figure V: siRNA α6 has no effect on ECFC apoptosis (A) or viability (B) after 96 hours of transfection. Results are mean + SEM of three different experiments.

A: Apoptosis. After 96 hours of transfection, cells were serum starved for 6 hours, lysed and used in a caspase 3 colorimetric assay.
There is no significant difference in caspase 3 activity between the different groups (untransfected ECFCs, ECFCs transfected with scramble siRNA or ECFCs transfected with siRNA α6). This result indicates that transfection with siRNA does not influence apoptosis.

B: Viability. 96 hours after transfection, dead and living cells were quantified using trypan blue. Transfection with siRNA α6 or scramble siRNA had no effect on cell viability, the number of dead and living cells was not significantly different in the different groups.

As a consequence, cellular effects observed on ECFCs transfected with siRNA α6 are caused by the reduction of α6 expression and not by an increased apoptosis or a decreased viability.
**Figure VI: expression of laminin in ischemic and non ischemic muscles**

Gastrocnemius sections were stained for mouse CD31 (green) and laminin (red). Laminin is a component of the basement membrane and of the extracellular matrix. Laminin is present between muscle fibers and all around blood vessels, in non ischemic muscles (A, C) as well as in ischemic muscles (B,D).
Figure VII: ECFCs recruited in ischemic muscles are colocalized with laminin.

Human ECFCs were identified with an anti-human CD31 antibody (red fluorescence). Laminin was stained with an anti-mouse laminin antibody (green fluorescence). Nuclei were stained with TOPRO3-iodide (represented in blue).

We did not find any ECFC in non ischemic muscles. In contrast, in ischemic muscles, where the basement membrane of damaged blood vessels may be exposed, ECFC are recruited and we can observe that they are colocalized with laminin.
Table I: SiRNA mix of human ITGA6 (ON-TARGETplus SMARTpool)

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Legends for video files

Injected human ECFCs, shown in red, are incorporated into the green mouse microvasculature of the ischemic muscle and form chimeric vessels. The nuclei are represented in blue.

References