β-Catenin Overexpression Augments Angiogenesis and Skeletal Muscle Regeneration Through Dual Mechanism of Vascular Endothelial Growth Factor–Mediated Endothelial Cell Proliferation and Progenitor Cell Mobilization

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Objective—β-Catenin plays a critical role in directing cell fate during embryogenesis, and uncontrollable activation leads to cancers, suggesting its importance in cell survival and proliferation. However, little is known regarding its role in endothelial cell (EC) and skeletal muscle proliferation and progenitor cell mobilization.

Methods and Results—β-Catenin enhanced ECs proliferation, protected ECs from apoptosis, and increased the capillary forming capabilities, which was completely blocked by inhibition of its nuclear translocation. In addition, the increased proliferation by β-catenin was associated with increased expression of cyclin E2. In skeletal myocytes, β-catenin overexpression increased proliferation with cyclin D1 expression, decreased apoptosis, and induced hypertrophy. Furthermore, β-catenin induced the expression of vascular endothelial growth factor (VEGF) in skeletal myocytes, resulting in EC proliferation. In a mouse hindlimb ischemia model, β-catenin significantly increased recovery of blood perfusion, capillary density along with enhanced VEGF expression, and the number of proliferating ECs and myocytes. Local delivery of β-catenin also promoted angiogenic progenitor cell mobilization and increased the number of satellite cells.

Conclusions—β-Catenin may be an important modulator of angiogenesis and myocyte regeneration not only by directly enhancing proliferation and survival of ECs and skeletal myocytes but also by inducing VEGF expression and promoting angiogenic progenitor cell mobilization and muscle progenitor cell activation. (Arterioscler Thromb Vasc Biol. 2006;26:91-98.)

Key Words: β-catenin ■ VEGF ■ angiogenesis ■ skeletal regeneration ■ progenitor cell

β-Catenin is an intracellular protein known to play dual roles in cells. In addition to its structural role in maintaining tissue architecture and cell polarity at adherens junctions, cytoplasmic β-catenin also translocates into the nucleus where it forms a complex with transcription factors of the Tcf/Lef family and activates the expression of specific genes involved in cell proliferation and survival.1,2 Although the critical role of β-catenin on the proliferative and migratory responses of cells during embryogenesis and in neoplastic disease have been well described previously, relatively little is known about the role of β-catenin on the endothelial cell (EC) in normal, controlled cell proliferation and migration.3,4 Recent data suggest that Wnt/β-catenin signaling may play a key role in vascular biology. For example, transfection of Wnt-1–expressing vector was shown to stimulate EC proliferation with β-catenin accumulation, which implies that Wnt proteins may regulate signal transduction in ECs via β-catenin.5 Furthermore, β-catenin was also identified in the cytoplasm of ECs of newly formed vessels around the area of infarction.6 In a recent study,7 the human vascular endothelial growth factor (VEGF) gene promoter has been reported to contain binding sites for β-catenin/Tcf, and the transfection of β-catenin to normal colon epithelial cells significantly increased VEGF expression. In addition, the Wnt pathway is also reported to play an important role in muscle regeneration.8

However, the downstream target genes and the exact mechanisms of the Wnt/β-catenin signaling pathway in ECs and skeletal muscle cells have not been clarified. Therefore, the aim of the present study was to evaluate the role of β-catenin in cell biological behaviors of ECs and skeletal myocytes and to elucidate the key signaling pathway of
β-catenin in these cells and crosstalk between the 2 types of cells. Furthermore, we investigated the role of β-catenin as a modulator of angiogenesis and myocyte regeneration in a murine hindlimb ischemia model.

Materials and Methods
Detailed material and methods are described in the Expanded Materials and Methods section (available online at http://atvb.ahajournals.org).

In Vitro Studies

Construction of Adenoviral Vectors Expressing Wild-Type β-Catenin
Adenoviruses expressing β-catenin constructs were produced using AdEasy kits (Q Biogene), and transfected cells were determined by the coexpression of green fluorescent protein (GFP).

Cell Culture and Adenoviral Transfection
Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth medium (Clonetics) as described previously. Four to six passage cells were used. To examine the effect of β-catenin, HUVECs were serum-starved for 15 hours, then treated with the indicated agents for 1 hour and stimulated with 2% FBS. For control studies, an adenoviral vector expressing only the GFP gene was used. Under these conditions, the transfection efficiency was >95%. C2C12 myoblasts (American Type Culture Collection) were cultured as described previously. Cells were maintained in growth medium (DMEM supplemented with 10% FBS, Gibco) and shifted to differentiation medium (DMEM supplemented with 5% heat-inactivated horse serum). For viral transfection, C2C12 cells were incubated with adenovirus (250 multiplicity of infection) in differentiation medium for 12 hours. Under these conditions, the transfection efficiency was >90%.

Inhibition of β-Catenin–Mediated Transactivation by Cadherin Derivatives
Dominant negative N-cadherin (NCadΔC), which lacks the extracellular domain, was used to suppress β-catenin–mediated transcriptional activity as described previously.

Immunoblot Analysis
Immunoblot assays were performed as described previously. The primary antibodies used were antitubal β-catenin antibody (1:500 dilution, Cell Signaling), anti-α-tubulin antibody (1:500 dilution, Oncogene), VEGF (1:500 dilution, Santa Cruz), cyclin D1 (1:500 dilution, Santa Cruz), and cyclin E2 (1:500 dilution, Santa Cruz). The secondary antibodies were antirabbit IgG/horseradish peroxidase (HRP) conjugate or antimouse IgG/HRP conjugate and antigoat IgG/HRP conjugate (1:2500 dilution, ECL, Amersham).

In Vivo Studies
Murine Hindlimb Ischemia Model
Male C57BL/6 (6 weeks old) mice were purchased from KBT Oriental Co Ltd (Charles River Grade, Tosa). All of the procedures were performed in accordance with the Institutional Animal Care and Use Committee of Seoul National University Hospital. To impair angiogenesis in response to hindlimb ischemia, mice were fed a 2% high-cholesterol diet. After 2 weeks of the high-cholesterol diet, unilateral limb ischemia was surgically induced in all of the animals. Under sufficient anesthesia with IP injection of a combination anesthetics (ketamine 50 mg/kg and xylazine 20 mg/kg, Bayer Korea), the entire left superficial femoral artery was ligated, cut, and excised. For gene therapy, 40 μL of vector solution (10^9 plaque forming units) was injected into 4 injection sites in the adductor and thigh muscles soon after the surgical procedure.

Progenitor Cell Mobilization: Fluorescence Activated Cell Sorter Analysis, ELISA, and Endothelial Progenitor Cell Culture
Peripheral blood was obtained from mice at 3 and 7 days after hindlimb ischemia. The VEGF concentration was measured using ELISA (R&D Systems). Fluorescence activated cell sorter (FACS) analysis was performed as described previously using CD34 (BD PharMingen) and Sca-1 (Biosource) antibodies. Endothelial progenitor cells (EPCs) were identified and calculated as described previously.

Statistical Analysis
All of the statistical analyses were performed using SPSS for Windows 10.0 (SPSS Inc). Continuous variables are expressed as mean ± SE and were analyzed by ANOVA test, using the Student-Newman-Keuls and Bonferroni post-hoc tests. All of the statistical analyses were 2-tailed, and P<0.05 was considered statistically significant.

Results
Effect of β-Catenin on EC Proliferation, Apoptosis, and Tube Formation
Fluorescent microscopy of HUVECs at 24 hours after transfection with adenovirus (25 multiplicities of infection) showed higher proliferative activity in cells transduced with the β-catenin gene. The proproliferative effect of β-catenin on ECs was more than twice that of GFP as quantified by WST-1 assay [absorption: 249.6±18.6 vs 100.0±3.74% for β-catenin wild-type (WT) vs GFP; P<0.05], which was completely inhibited by NCadΔC. In addition, under serum-deprived conditions, the transfection of β-catenin WT to ECs significantly reduced the subdiploid apoptotic fraction of DNA as measured by FACS analysis compared with GFP-transduced cells, which was reversed by NCadΔC, suggesting that the antiapoptotic effects were mediated by the transcriptional activity of β-catenin. Furthermore, EC function as measured by Matrigel tube formation was significantly better in β-catenin WT-transduced cells compared with GFP controls (tube length relative to GFP control [%]: 258.2±31.8% in β-catenin WT; P<0.05; Figure I, available online at http://atvb.ahajournals.org).

Effect of β-Catenin on Skeletal Myocyte Proliferation, Apoptosis, and Hypertrophy
Adenovirus-mediated β-catenin overexpression in skeletal myocytes after differentiation induced hypertrophy, that is, an increase in myocyte size (mean area and width). In addition, β-catenin transfection resulted in enhanced proliferation and resistance to serum-deprived apoptosis (Figure II, available online at http://atvb.ahajournals.org). All of these effects in the skeletal myocyte were inhibited by adding NCadΔC, suggesting the importance of the transcriptional activation of β-catenin in myocyte hypertrophy, proliferation, and resistance to apoptosis.

Dual Mechanism of EC Regulation by β-Catenin
To investigate downstream target signals of β-catenin in EC, major cell-cycle regulators, cyclin E2 and cyclin D1, were examined. Cyclin E2 expression was consistently increased after β-catenin transduction, whereas no significant change was observed in cyclin D1 expression (Figure 1A). To
confirm the cell biological significance of cyclin E2, cell cycle analysis was performed using flow cytometry. As expected, β-catenin WT decreased the percentage of cells in the G1 phase and increased the number of cells in the S phase, a profile that is typically associated with acceleration of G1 (Figure 1B). In addition, the increased expression of cyclin E2 with β-catenin WT was inhibited by NCadΔC, which suggests that β-catenin enhances cyclin E2 expression after its nuclear translocation (Figure 1C).

Because VEGF is a major cytokine involved in EC proliferation, survival, and angiogenesis and was recently discovered as a downstream molecule controlled by β-catenin in colon cancer, we hypothesized that β-catenin, in addition to the direct prosurvival and antiapoptotic effects, may have indirect effects on EC through VEGF secretion by other surrounding cells. Therefore, we targeted skeletal myocytes and examined the effects of β-catenin transduction in myocytes with regard to VEGF expression. After β-catenin transfection, VEGF expression was markedly increased in myocytes and reversed by NCadΔC (Figure 1D). To validate the hypothesis that β-catenin promotes EC proliferation by way of VEGF expression, an EC survival assay was performed using the supernatant from β-catenin–stimulated myocyte culture and blocking the antibody for VEGF. The WST-1 assay showed a significant increase of EC proliferation after adding the supernatant of C2C12 cell culture to HUVECs, which was reversed by adding anti-VEGF neutralizing antibody (Figure 1E), suggesting that the increased survival from the addition of supernatant from β-catenin–stimulated myocyte culture was through VEGF.

**β-Catenin Promotes Angiogenesis and Myocyte Regeneration in a Mouse Hindlimb Ischemia Model**

To investigate the in vivo effects of β-catenin on angiogenesis and skeletal muscle regeneration, we used a mouse hindlimb ischemia model. First, to confirm that ischemia induces β-catenin, we examined the expression of β-catenin in ischemic hindlimb in a baseline experiment, which showed that the expression of β-catenin was significantly increased in the ischemic limb compared with the nonischemic limb (Figure 2A). Tissue ischemia induced the expression of β-catenin and VEGF, but this phenomenon was only transient and decreased after day 3. However, when the β-catenin gene was delivered by adenoviral vector, we found that β-catenin and VEGF expression was stronger with prolonged expression up to day 5 (Figure 2B). Serial laser Doppler perfusion imaging of the ischemic left hindlimb showed that recovery of blood flow was faster and more intense in the β-catenin gene–delivered group than in the control group (Figure 2C). By day 14, the ratio of ischemic/nonischemic blood flow was significantly greater in the β-catenin WT group (0.93±0.05 versus 0.70±0.03 for β-catenin WT versus GFP group; P<0.01; Figure 2D).

Immunohistochemical staining for the EC marker PECAM-1 was performed on skeletal muscle sections re-
Hindlimb Ischemia Model

Activates Skeletal Progenitor Cells in a Mouse

Mobilization of Angiogenic Progenitor Cells and

Expression of β-catenin in ischemic limb, and expressed in both skeletal myocytes and ECs (Figure 3C).

VEGF expression in skeletal muscle were also significantly increased in the β-catenin group compared with the GFP control group at day 5 (Figure 5A). We also observed increased skeletal muscle regeneration, as shown by increased regenerating myocytes (small cells with central nuclei), in the β-catenin group (Figure 5C).

Local β-Catenin Gene Transfer Promotes Mobilization of Angiogenic Progenitor Cells and Activates Skeletal Progenitor Cells in a Mouse Hindlimb Ischemia Model

Furthermore, to investigate the role of β-catenin in progenitor cell mobilization, peripheral blood mononuclear cells were isolated for FACS analysis. We found a greater fraction of CD34-positive and Sca1-positive cells in the β-catenin group (Figure 6A). To characterize the mobilized progenitor cell after local β-catenin gene transfer, the number of cells uptaking DiI-AcLDL among the double-positive (CD34 and Sca1) cells was counted in peripheral blood mononuclear cells after flow cytometry. More EPCs uptaking DiI-acetylated LDL (DiI-AcLDL) were observed in the β-catenin group (Figure 6B). To find out the underlying mechanism of progenitor cell mobilization, we measured the concentration of VEGF in plasma, which showed an increased level of VEGF in the β-catenin group at day 3 (Figure 6C). We also evaluated the potential effect of β-catenin gene transfer to muscle on the skeletal progenitor cell. Gene transfer of β-catenin to muscle increased both CD34/Sca1 double-positive cells (Figure 6C) and the number of satellite cells in muscle analyzed by skeletal myocyte single-fiber culture technique (Figure 6E; 4',6-diamidino-2-phenylindole–positive satellite cells per muscle fiber; 4.4±2.9 in GFP versus 10.1±2.7 in β-catenin; *P<0.05), suggesting that local gene transfer of β-catenin may have also increased skeletal progenitor cells in muscle.

Discussion

In this study, we showed that overexpression of β-catenin led to enhanced EC survival, function, and proliferation. Furthermore, these prosurvival effects were mediated through cyclin E2, which is a novel downstream target molecule of β-catenin in ECs that was not reported previously in other studies. In addition, we found that β-catenin overexpression not only enhances proliferation and inhibits apoptosis of skeletal
myocytes, but also increases VEGF expression in skeletal myocytes, suggesting a paracrine effect of β-catenin on ECs via surrounding myocytes. All of these effects were inhibited by NCadΔC, suggesting that these effects are, indeed, mediated by the transcriptional activity of β-catenin.

In vivo, critical ischemia led to a transiently increased expression of β-catenin, which suggests that β-catenin may be the actual modulator of angiogenesis in ischemic tissue. Accordingly, adenovirus-mediated β-catenin gene transfer resulted in a sustained increase in β-catenin, as well as VEGF expression in ischemic hindlimb muscles, as shown in Figure 4. Enhanced expression of VEGF and cell cycle-related genes, such as cyclin D1 and cyclin E2, was observed in β-catenin-overexpressing muscles. Double immunohistochemical staining for VEGF and cyclin D1 showed that the VEGF-secreting cell was identical to the β-catenin-overexpressing cell.

Figure 3. Effect of β-catenin gene transfer on the ratio of capillary:muscle fiber. A, Immunohistochemistry for PECAM-1 in ischemic limb tissues at postoperative day 14. PECAM-1–positive cells were counted in 10 different microscopic fields of ≥3 different sections from each animal under light microscopy. There were more capillaries positive for PECAM-1 (brown) in the β-catenin WT group than in the GFP group. Scale bars=50 μm. B, An increase in the capillary density and capillary:myocyte ratio could be observed in the β-catenin WT group than in the GFP group (*P<0.01, n=5 in both groups). C, To identify the β-catenin–expressing cells after adenovirus-mediated gene transduction, double staining of hemagglutinin (HA, tagged to the adenoviral vector, indicating exogenous β-catenin) and PECAM-1 (red) for endothelial cell or MHC (green) for myocytes were performed. β-Catenin was expressed in both skeletal myocyte and endothelial cell.

Figure 4. β-Catenin promotes angiogenesis via VEGF expression in ischemic hindlimb. A, Immunohistochemistry for VEGF in hindlimb ischemic tissue at postoperative day 3. Enhanced expression of VEGF was observed in β-catenin group, suggesting that β-catenin gene transfer augmented ischemia-induced VEGF expression. Scale bars=50 μm. B, Gene transfer with β-catenin augmented ischemia-induced VEGF expression in ischemic limb, with enhanced expression of cell cycle–related gene. Immunoblot analysis for VEGF, cyclin D1, and cyclin E2 in mouse hindlimb muscle 3 days after tissue ischemia showing increased expression of VEGF and cycline D1 mainly from skeletal muscle. C, Double immunohistochemical staining was performed to identify the VEGF-secreting cell in hindlimb ischemic tissue, which showed that VEGF-expressing cell is identical to β-catenin overexpressing cells.
expression, leading to a significant augmentation of angiogenesis and myocyte regeneration in a mouse hindlimb ischemia model. In addition, β-catenin increased the mobilization of hematopoietic progenitor cells from the bone marrow and the number of satellite cells. These are all novel findings, which have not been reported previously.

The Wnt signaling pathway is involved in the control of multiple cellular processes. Recent studies have demonstrated the expression of Wnt ligands, Wnt receptors, and Wnt inhibitors in vascular cells. However, the specific effect of β-catenin overexpression on EC survival and function and, furthermore, on new vessel formation have not been studied previously. Our results provide new insight into a possible role of the Wnt/β-catenin signaling pathway in angiogenesis.

In the present study, we showed that in ECs, in contrast to other cancer cells, the expression of cyclin E2 rather than cyclin D1 is significantly increased, which leads to the propagation of the cell cycle from the G1 phase to the S phase. Previously, it was reported that cyclin D1 is a direct target of the Tcf/LEF-1 pathway through a binding site in the cyclin D1 promoter region in the colon cancer cell. The same finding was observed when we transferred the β-catenin gene in skeletal muscle cells in vitro and limb muscle in vivo. In ECs, however, we observed increased expression of cyclin E2 rather than cyclin D1. The finding that β-catenin increases cyclin E2 expression in ECs is a novel one, which needs to be additionally studied to understand how β-catenin controls the EC growth at a molecular level.

The modulations of β-catenin on ECs were reversed by NCadΔC. This suggests that the binding of NCadΔC to β-catenin may compete with other transcription factors that interact with β-catenin. It may be deduced, therefore, that the binding of β-catenin to dominant-negative cadherin, which has a truncated extracellular domain with an intact cytoplasmic tail, may have inhibited the binding of β-catenin to other transcription factors, such as the Tcf/Lef family, and blocked its transcriptional activity.

Another key finding of the present study is that β-catenin, in addition to the direct pro-survival effects, has an indirect effect on ECs via myocytes, which plays a critical role in angiogenesis. We found that β-catenin gene transfer on skeletal myocytes leads to a significant increase in VEGF expression. Furthermore, the importance of this so-called “paracrine” effect on EC proliferation and angiogenesis was confirmed in experiments where an antibody against VEGF resulted in partially decreased proliferation in vitro and decreased new vessel formation in vivo. These findings suggest the Wnt/β-catenin pathway may play a key role in angiogenesis both directly, by enhancing EC proliferation and function, and indirectly, by inducing the expression of proangiogenic molecules in surrounding cells.

In addition to the paracrine effects, β-catenin enhances proliferation and reduces serum deprivation–induced apoptosis in myocytes. β-Catenin overexpression also increased the mean area and size of the myocyte. These findings are compatible with previous reports showing that stabilization of β-catenin in cardiomyocytes is necessary for the hypertrophic response.

In a hindlimb ischemia model, we observed a significantly increased but transient expression of β-catenin after the induction of ischemia, suggesting that β-catenin is a gene that responds to ischemic insult and may be involved in angiogenesis. This hypothesis was confirmed by showing that the overexpression β-catenin in the ischemic tissue by gene transfer resulted in sustained increased expression of β-catenin, leading to significantly augmented angiogenesis and recovery of blood flow. Our results also suggest that the impact of β-catenin on angiogenesis in ischemic tissue may be greater than other angiogenic molecules because of its dual effect on both EC and myocytes. On top of its direct proliferative role, β-catenin also enhances VEGF expression in gene-transfected skeletal muscle, which leads to the increased plasma level of VEGF. The increased concentration of VEGF at local muscle may help ECs proliferate and survive, leading to angiogenesis, and the increased VEGF in
circulation may lead to progenitor cell mobilization, mainly hematopoietic stem cells from bone marrow, which might augment the angiogenic effect of local β-catenin gene transfer to ischemic hind limb.

Furthermore, there have been several reports regarding the roles of β-catenin in skeletal myogenesis and regeneration. In this study, we observed that β-catenin increased CD34 and Sca1 double-positive muscular stem cells, which were reported to have an important role in skeletal regeneration. Although it is not confirmed whether the muscular stem cells are derived from the ones in muscle or from mobilized hematopoietic progenitor cells, it is possible that these progenitor cells have an important role in tissue repair of the ischemic hindlimb. Considering that the number of skeletal satellite cells significantly increased after β-catenin gene transfer, we can think that stimulatory effect of β-catenin on skeletal progenitor cells contributed to the accelerated regeneration of skeletal muscle.

There are previous reports showing induction of inflammation by adenovirus mediated gene transfection which may have led to the increased VEGF and augmented angiogenesis. However, our study showed the clear benefit of β-catenin over GFP, both using the adenoviral transfection technique. Thus, we believe that the enhanced angiogenic potential we observed in the present study was because of β-catenin and not inflammation from adenoviral transfection.

In conclusion, we show for the first time that β-catenin directly increases proliferation of ECs through cell cycle propagation and indirectly enhances EC survival by inducing VEGF expression from surrounding myocytes. Furthermore, β-catenin gene transfer significantly induced the mobilization of angiogenic progenitor cells in the circulating blood and

Figure 6. β-Catenin promotes mobilization of progenitor cell from bone marrow and increases the number of satellite cells. A, Local delivery of β-catenin gene promotes progenitor cell mobilization from bone marrow. FACS analysis of peripheral blood mononuclear cells shows increased CD34 and Sca1 double-positive cells in the β-catenin group (PBMNC indicates peripheral blood mononuclear cell). B, Presumable EPCs uptaking DiI-AcLDL were counted in peripheral blood mononuclear cells with flow cytometry. Local gene transfer of β-catenin increased the circulating EPCs uptaking AcLDL compared with control GFP. C, VEGF concentration in the peripheral blood was measured by ELISA at 3 days after femoral artery ligation. Local gene transfer of β-catenin augmented ischemia-induced increase of plasma VEGF concentration compared with GFP gene transfer. In β-catenin group, increased VEGF concentration was identified. However, there was no significant increase in circulating VEGF concentration in the GFP group compared with the ischemia only group (PB indicates peripheral blood). D, After β-catenin gene delivery to skeletal muscle, single fibers of skeletal myocytes were cultured from that muscle and analyzed by FACS. Increased proportion of CD34 and Sca1 double-positive cell, presumable progenitor cells in muscle, was identified in the β-catenin group compared with GFP control group. E, Skeletal muscle satellite cells were counted with 4',6-diamidino-2-phenylindole staining after isolation from the gene-transfected muscles. The number of satellite cells was higher in the β-catenin group compared with GFP control group.
increased the number of skeletal muscle progenitor cells in transfected muscle, leading to enhanced angiogenesis and muscle regeneration in a mouse hindlimb ischemia model. These data suggest that β-catenin may be an important regulator of angiogenesis and skeletal muscle regeneration in ischemic tissue.

Acknowledgments
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References
Expanded Material and Methods

Construction of adenoviral vectors expressing wild type (WT) β-catenin

Human wild type β-catenin (β-Catenin WT) plasmid, a generous gift from Dr. Bert Vogelstein of Johns Hopkins University, USA, was subcloned into a shuttle vector (pAdTrack-CMV). The recombinant shuttle vector was co-transfected with adenoviral genome (pAdEasy-1) into E-coli (BJ5183) where homologous recombination occurred. The recombinant Adeno-β-catenin was transfected to 293 cells to amplify viral particles, which were purified by CsCl ultracentrifugation and dialysis. Successful construction of the new adenoviral vector was confirmed by immunoblot analysis, and transfected cells were determined by the co-expression of green fluorescent protein (GFP).

Construction of Dominant negative N-cadherin (NCadΔC)

The disruption of N-cadherin by truncation of its extracellular domain has been suggested to sequester β-catenin in the plasma membrane thereby inhibiting its intranuclear transcriptional activity.\(^1\),\(^2\) The NCadΔC construct was obtained with the permission of Dr. Jeffery Gordon (Washington University, St. Louis, MO). NcadΔC was inserted into the BglII/EcoRI site of pMSCV-IRES-GFP retroviral vector, giving
pMSCV-NcadΔC-IRES-GFP. 293T cells were transfected with the retroviral vectors pMSCV-NcadΔC-IRES-HA-GFP or control empty vector, pMSCV-IRES-GFP, using LipofectAMINE Plus reagents (Invitrogen, Carlsbad, CA). Viral supernatant was collected 48h later, centrifuged at 1,000 x g for 5 min, and stored at -80°C.

Cell viability: WST-1 assay

HUVECs in 96-well plates were infected with adenoviral (25 m.o.i.) and retroviral vectors. After incubation for 24 and 48 hours, 10µl/well of cell proliferation reagent WST-1 (Roche molecular biochemicals) were added and incubated for another 4 hours in the same incubator. After shaking thoroughly for 5 seconds on a shaker, absorbance was measured at 450 nm. To investigate the effect of β-catenin on serum-deprivation-induced apoptosis of HUVECs, WST-1 analysis was performed on HUVECs 2-4 days after β-catenin and retroviral transfection in EGM without adding FBS. Each experiment was repeated 12 times.

Cell cycle analysis and apoptosis: Fluorescence Activated Cell Sorter (FACS) analysis

DNA fragmentation was assessed by flow cytometry. For these assays, cells were transfected and serum-starved for 2-4 days. At several time points after serum starvation,
the attached and floating cells were harvested and fixed in cold 90% ethanol for at least 3 hr and then resuspended in staining buffer consisting of 1 mg/ml RNaseA, 20 µg/ml propidium iodide, and 0.01% Nonidet P-40. DNA content was analyzed by flow cytometry on the FL-2 channel, and gating was set to exclude debris and cellular aggregates. Flow cytometric analysis was performed on a FACStar Plus (Becton Dickinson, Heidelberg, Germany).

**Tube formation assay by Matrigel**

Tube formation assay and quantification were performed as described previously, using a minimal volume of Matrigel. For quantification of tube formation, the total length of the tubes formed in a unit area was measured by Image-Pro Plus software (Mediacybernetics, USA). For each test, five randomly chosen areas were measured and averaged.

**Effect of β-catenin on skeletal myocyte hypertrophy**

Myocyte analysis was performed as described previously. Myocytes expressing β-catenin were identified by GFP expression and only transfected myocytes were assessed for hypertrophy. Myocyte area and diameter were quantified as follows: 10 fields were
chosen randomly, and approximately 10 myocytes were measured per field. The average diameter per myocyte was calculated as the mean of ten measurements taken along the length of the myocyte.

*Effect of conditioned media from β-catenin transfected C2C12 on ECs proliferation*

To evaluate the role of β-catenin on myocytes in inducing angiogenic molecules such as VEGF, adenovirus expressing β-catenin or GFP were transfected to C2C12 cells after differentiation. Twenty-four hours later, the supernatant of culture medium was aspirated and centrifuged. The resulting conditioned media were collected and used for the bioassay to study their effect on EC proliferation using WST-1 assay. In addition, to examine the paracrine effect of β-catenin on ECs via myocytes, VEGF neutralizing antibody (R&D Systems) was added to the culture medium at 4µg/mL.

*Laser Doppler perfusion images*

Laser Doppler perfusion imaging (LDPI, Moor Instruments) was used to record serial blood flow measurements over the course of 2 weeks postoperatively. To minimize data variability due to ambient light and temperature, the LDPI index was expressed as the ratio of ischemic to nonischemic limb blood flow.
Histological analysis and immunohistochemistry

Mice were euthanized with an overdose of sodium pentobarbital. Medial thigh adductor muscle of the ischemic (left) and nonischemic (right) limbs were harvested and processed for histological analyses. Capillary densities of both ischemic and nonischemic skeletal muscle tissues, at 14 days after surgery, were immunohistochemically determined, using anti-murine platelet-endothelial cell adhesion molecule (PECAM)-1 monoclonal antibody (B&D Pharmingen).

Single fiber culture and satellite cell count

Single fibers from the ischemic hindlimb muscles were isolated at 3 and 7 days after β-catenin gene delivery and satellite cells were counted as described previously.6, 7 Isolation and FACS analysis of muscle-derived cell were performed as previously described.8


**Cell FACS: Survival Assay**

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**Tube formation assay**

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Fig II.

Control + GFP  
Control + β-catenin WT  
NCadΔC + β-catenin WT

**Myocyte mean area (Arbitrary unit)**

- Control + GFP: 4.99%
- Control + β-catenin WT: 25.81%
- NCadΔC + β-catenin WT: 13.79%

**Cell FACS: Survival Assay**

- Control + GFP: 13.79%
- Control + β-catenin WT: 4.99%
- NCadΔC + β-catenin WT: 25.81%