Transplantation of Mesenchymal Cells Rejuvenated by the Overexpression of Telomerase and Myocardin Promotes Revascularization and Tissue Repair in a Murine Model of Hindlimb Ischemia

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Rationale: The number and function of stem cells decline with aging, reducing the ability of stem cells to contribute to endogenous repair processes. The repair capacity of stem cells in older individuals may be improved by genetically reprogramming the stem cells to exhibit delayed senescence and enhanced regenerative properties.

Objective: We examined whether the overexpression of myocardin (MYOCD) and telomerase reverse transcriptase (TERT) enhanced the survival, growth, and myogenic differentiation of mesenchymal stromal cells (MSCs) isolated from adipose or bone marrow tissues of aged mice. We also examined the therapeutic efficacy of transplanted MSCs overexpressing MYOCD and TERT in a murine model of hindlimb ischemia.

Methods and Results: MSCs from adipose or bone marrow tissues of young (1 month old) and aged (12 months old) male C57BL/6 and apolipoprotein E–null mice were transiently transduced with lentiviral vectors encoding TERT, MYOCD, or both TERT and MYOCD. Flow cytometry and bromodeoxyuridine cell proliferation assays showed that transduction with TERT and, to a lesser extent, MYOCD, increased MSC viability and proliferation. In colony-forming assays, MSCs overexpressing TERT and MYOCD were more clonogenic than mock-transduced MSCs. Fas-induced apoptosis was inhibited in MSCs overexpressing MYOCD or TERT. When compared with aged mock-transduced MSCs, aged MSCs overexpressing TERT, MYOCD, or both TERT and MYOCD increased myogenic marker expression, blood flow, and arteriogenesis when transplanted into the ischemic hindlimbs of apolipoprotein E–null mice.

Conclusions: The delivery of the TERT and MYOCD genes into MSCs may have therapeutic applications for restoring, or rejuvenating, aged MSCs from adipose and bone marrow tissues. (Circ Res. 2013;113:902-914.)

Key Words: adipose–derived mesenchymal stem cells ■ aging ■ bone marrow stem cells ■ hindlimb ischemia ■ mesenchymal stem cells ■ myocardin ■ telomerase

The capacity of organs to self-repair decreases with age, possibly resulting from the reduced functional capabilities of stem cells.1 Mesenchymal stromal cells (MSCs) are a population of stem cells derived from bone marrow (BM) or adipose tissue (AT). Like other stem cells, MSCs are susceptible to age-related changes, including increased rates of apoptosis and senescence,2–4 which reduce their ability to contribute to endogenous repair processes.5 Furthermore, as an individual ages, the effectiveness of their MSCs after transplantation diminishes.6

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Aging is a major risk factor for cardiovascular disease. Furthermore, with the onset of age-related cardiovascular disease, which often occurs secondarily to atherosclerotic plaque–induced vessel narrowing, the function of both resident and circulating stem/progenitor cells is diminished.24 The combination of these disease-related and age-related deficits may contribute to decreased muscle and vessel regeneration after injury and facilitate the development of atherosclerosis and its sequelae in older individuals.1,10 Therefore, an appropriate therapy for age-related vascular disease may be to replenish stem cell function by rejuvenating existing cells or by transplanting stem/progenitor cells that will supply the ischemic tissue with new vessels to prevent ischemic tissue damage.11–13

Cells derived from AT contain a population of adult multipotent mesenchymal stem cells that can regenerate damaged...
cardiovascular tissues. Recently, we identified a subpopulation of AT–derived MSCs (AT-MSCs) that expresses high levels of the catalytic subunit of telomerase (ie, telomerase reverse transcriptase or TERT) and myocardin (MYOCD). MYOCD is a key regulator of cardiovascular myogenic development and acts as a nuclear transcription cofactor for myogenic genes, as well as genes involved in muscle regeneration and protection against apoptosis. Telomerase maintains telomere length, contributes to cell survival and proliferation, and prevents cellular senescence. We have shown that AT-MSCs that coexpress TERT and MYOCD have increased endogenous levels of octamer-binding transcription factor 4, MYOCD, myocyte-specific enhancer factor 2c, and homeobox protein NKx2.5. These observations suggest that TERT and MYOCD may act together to enhance cardiovascular myogenic development. In the present study, we examined the interplay between TERT and MYOCD and each of their roles in the conversion of aged MSCs to rejuvenated antiapoptotic, promyogenic stem cells. We show that the delivery of the TERT and MYOCD genes can restore MSCs from aged mice by increasing cell survival, proliferation, and smooth muscle myogenic differentiation in vitro. Furthermore, we demonstrate the therapeutic efficacy of these rejuvenated cells in an in vivo hindlimb ischemia model.

Methods

Animal Care
All procedures were approved by the Institutional Ethics Committee for animal research. All studies conform to either the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health or the Directive 2010/63/EU of the European Parliament.

Isolation of MSCs and Cell Culture
For our experimental studies, we used 1- and 12-month-old male C57/B6 mice, and ApoE−/− mice and from 12-month-old GFP-expressing mice by using a modified version of the protocol described by Zuck et al. The vascular stromal fraction was plated, and AT-MSCs were selected on the basis of their plastic adherence. Before transduction, AT-MSCs from 1- and 12-month-old male C57 and 12-month-old male GFP-expressing mice (GFP+ AT-MSCs) were cultured for up to 3 passages and characterized for the expression of markers for mesenchymal stem cells, endothelial cells, fibroblasts, smooth muscle cells, and monoocyte/macrophages. Experiments were also performed with passage 3 BM-MSCs isolated from 12-month-old male C57 mice and passage 3 human BM mesenchymal stem cells, used as controls (Lonza, Atlanta, GA). Detailed methods are described in the Online Data Supplement.

Fluorescence-Activated Cell Sorting (FACS) and Western Blot Analysis of Transduced Cells
Murine AT-MSCs, murine BM-MSCs, or human BM mesenchymal stem cells (1×10^6 cells) were plated in 60-mm culture dishes. Serial dilutions of concentrated lentiviral supernatants were incubated with cells for 16 hours in a volume of 10 mL in the presence of polybrene (16 µg/mL). Cells were maintained in culture for 5 days, trypsinized, and analyzed for YFP-TERT expression with the BD FACS Canto II (BD Biosciences, San Jose, CA) equipped with a 488-nm laser (for excitation of YFP, 517 nm). Data were analyzed by using FACS Diva software (BD Biosciences), and the percentage of transduced cells was represented by the percentage of YFP-positive cells. MYOCD-V5 expression was analyzed by means of Western blot analysis.

Colony-Forming Unit and Cell Proliferation Assays
For the colony-forming unit assay, nontransduced, mock-transduced, or lentivirus-transduced AT-MSCs from 1- and 12-month-old C57 and ApoE−/− mice were trypsinized and introduced into methylcellulose medium (MethoCult MG3534, StemCell Technologies, Vancouver, BC, Canada) at a density of 1.5×10^5 cells/cm^2 by single-cell plating. Plates were examined under phase-contrast microscopy, and colonies were scored after 14 days from triplicate cultures. The number and size (diameter) of colony-forming units were determined by counting 8 different high-power fields by using a 10× objective. Fields for counting colony-forming units were randomly located at half-radius distance from the center of the monolayers. For the proliferation assay, murine wild-type or lentivirus-transduced AT-MSCs (1×10^4 cells/cm^2) were plated in a 96-well plate and counted daily from day 1 to day 5. At each time point, the population doubling time was calculated by using the following equation: $t = \frac{\log_{10} \left[ \frac{N}{N_0} \right]}{3.33}$, where $N$ is the total number of cells, and $N_0$ is the number of seeded cells.

Brdu Cell Proliferation Assay
Cell proliferation was quantified on the basis of bromodeoxyuridine (Brdu) incorporation in nontransduced AT-MSCs, mock-transduced AT-MSCs, and AT-MSCs transduced with TERT, MYOCD, or both TERT and MYOCD. Passage 3 cells (2×10^4 cells/mL) were plated in 96-well plates. Twenty-four hours before cells were harvested, media were replaced with serum-free media containing 10 µmol/L Brdu (Calbiochem, La Jolla, CA). At the time of harvest, cells were trypsinized, washed with phosphate-buffered saline (PBS),
Cytotoxicity Testing and Annexin V and Propidium Iodide Viability Assays
A live/dead viability/cytotoxicity kit containing SYTOX Red (Invitrogen) was used to measure the cytotoxicity of lentiviral transduction. Data were analyzed by using FACS Diva software (BD Biosciences), and the percentage of dead cells was represented by the percentage of SYTOX Red–positive cells. For the Annexin V/propidium iodide (PI) viability assay, 1×10⁴ murine wild-type or lentivirus-transduced BM-MSCs (mock or TERT/MYOCD–transduced cells) were plated in 60-mm culture dishes under serum-starved culture conditions (Dulbecco’s Modified Eagle Medium and 2% fetal calf serum) and treated overnight with FasL (500 ng/mL). The Annexin V–Fluorescein Isothiocyanate Kit from Pharmingen (Franklin Lakes, NJ) was used to measure cell viability. Data were analyzed by using FACS Diva software (BD Biosciences). Detailed methods are described in the Online Data Supplement.

Osteogenic, Adipogenic, and Myogenic Differentiation Assays
For osteogenic differentiation analysis, we examined Alizarin Red S staining in nontransduced, mock-transduced, MYOCD-transduced, TERT-transduced, and MYOCD+TERT–transduced AT-MSCs from 1- and 12-month-old C57 and ApoE⁻/⁻ mice that were cultured for 21 days in osteogenic differentiation medium (STEMPRO Osteogenesis Differentiation Kit, Invitrogen). The extracted stain was then transferred to a 96-well plate, and the absorbance was measured at 450 nm using a SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp, Sunnyvale, CA).

For adipogenic differentiation analysis, we examined Oil Red O staining in nontransduced, mock-transduced, MYOCD-transduced, TERT-transduced, and MYOCD+TERT–transduced AT-MSCs from 1- and 12-month-old C57 and ApoE⁻/⁻ mice that were cultured for 14 days in adipogenic differentiation medium (STEMPRO Adipogenesis Differentiation Kit, Invitrogen). The extracted stain was then transferred to a 96-well plate, and the absorbance was measured at 450 nm by using a SpectraMax 340 plate reader/spectrophotometer. Data were analyzed by using FACS Diva software (BD Biosciences). Detailed methods are described in the Online Data Supplement.

Vascular Endothelial Growth Factor Expression
Forty-eight hours after the final change of fresh medium, supernatants were collected from nontransduced, mock-transduced, MYOCD-transduced, TERT-transduced, and MYOCD+TERT–transduced AT-MSCs from 1- and 12-month-old C57 and ApoE⁻/⁻ mice (n=5 mice per group), and cells were harvested. Western blot with anti–endothelial growth factor (VEGF) and anti–β-actin antibody (Sigma) was performed to analyze the concentration of murine vascular endothelial growth factor (VEGF) in cellular extracts and supernatants by using an enzyme-linked immunosorbent assay (Quantikine Murine VEGF Immunoassay, R&D Systems, Minneapolis, MN). Detailed methods are described in the Online Data Supplement.

Unilateral Hindlimb Ischemia
Unilateral hindlimb ischemia was induced in 12-month-old male ApoE⁻/⁻ mice (weighting 25–30 g) by ligation of the proximal left femoral artery and vein. One day after femoral ligation, mice (n=5 per group) were randomly assigned to receive a single dose of 1 of the following: (1) mock-transduced allogeneic GFP+ AT-MSCs (from 12-month-old GFP mice; 3×10⁴ cells/500 µL); (2) allogeneic GFP+ AT-MSCs (from 12-month-old GFP mice) transduced with TERT and MYOCD (3×10⁴ cells/500 µL); or (3) PBS (500 µL) as a noncellular control. Blood flow was measured in anesthetized animals 1 day before ligation (baseline), 1 day after ligation (preinjection), and at 2 weeks after injection by using a Laser Doppler Perfusion Imager System (PIM II, Perimed, Ardomar, PA). Blood flow was also measured in nonligated 12-month-old C57 mice (n=3). Detailed methods are described in the Online Data Supplement.

Histological Evaluation of Capillary and Arteriolar Density and Cell Proliferation
On day 21 after the injection of PBS or cells, the ischemic and nonischemic limbs of each 12-month-old ApoE⁻/⁻ mouse were removed. Each hindlimb was transversely cut into 5 equal sections (proximal to distal) and embedded in 5 separate paraffin blocks. For histological evaluation of capillaries and arterioles and on cell proliferation in vivo by performing immunohistochemical analysis of von Willebrand factor, smooth muscle α-actin, and Ki-67 in 5-μm thick paraffin-embedded tissue sections of adductor and semimembranosus muscles. Detailed methods are described in the Online Data Supplement.

Immunofluorescence Studies and Determination of Cell Engraftment Rate
At 21 days after the injection of PBS or GFP+ AT-MSCs (mock-transduced or transduced with TERT and MYOCD) into the ischemic hindlimbs of ApoE⁻/⁻ mice, the hindlimbs were harvested for immunofluorescence staining of smooth muscle α-actin with phycoerythrin-conjugated antirabbit IgG (Invitrogen). The number and distribution of mock-transduced AT-MSCs or AT-MSCs transduced with TERT and MYOCD were determined by counting cells positive for GFP and 4′,6-diamidino-2-phenylindole (DAPI) staining. The number and location of smooth muscle α-actin–positive cells were determined by counting cells positive for phycocerythrin. Detailed methods are described in the Online Data Supplement.

Western Blots
Total proteins from MSCs or from ischemic and nonischemic skeletal muscle tissues of injected mice were isolated in ice-cold radioimmunoprecipitation buffer (Sigma Aldrich). Proteins were separated under reducing conditions and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). After blocking, the membranes were incubated overnight at 4°C with primary antibodies against 1 of the following: (1) MYOCD (monoclonal mouse IgG2B clone, R&D Systems), (2) Annexin V (BD Biosciences), (3) cardiac actin (Sigma Aldrich), (4) smooth muscle α-actin (Sigma Aldrich), (5) V5-epitope (Invitrogen), (6) caspase-3 (Cell Signaling, Boston, MA), or (7) cleaved caspase-3 (Cell Signaling). Equal loading and protein transfer were verified by stripping and reprobing each blot with anti–β-actin or anti-GAPDH antibody (Sigma).

Statistical Analysis
Data were expressed as the mean±SD. Two-group comparisons were performed by using a Student t test for paired values. Multiple-group comparisons were performed by using ANOVA and the Mann–Whitney post hoc test to determine statistical significance within and between groups (GraphPad Prism 5). A P value <0.05 was considered significant.

Results
Murine AT-MSCs Express Mesenchymal Stem Cell Markers
At a low passage number (ie, P3), a considerable number of AT-MSCs in primary culture expressed endothelial
progenitor cell markers (CD45-CD34-CD133−: 37±24%; CD45-CD133+CD34−: 2±3%; CD45-CD34+CD133−: 6±3%), took up Dil (1,1′dioctadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate)-labeled acetylated low-density lipoprotein in vitro, or expressed smooth muscle or pericyte markers (smooth muscle α-actin: 47.9±4%; desmin: 7.4±5.0%, respectively) (data not shown). In contrast, only a few AT-MSCs expressed mesenchymal stem cell markers (CD105: 4.8±0.2%; CD44: 60±4%; CD29: 1.4±0.5%; CD71: 0.2±0.01%; CD106: 0.01±0.00%). At higher passages in culture (P>3), only a small fraction of adherent cells expressed endothelial progenitor cell or endothelial cell markers (CD31 and CD34), whereas the majority expressed mesenchymal stem cell markers (data not shown). These findings are in agreement with our previous study. Therefore, unless otherwise indicated, AT-MSCs were used at P>3 in all experiments.

**Lentivirus-Transduced AT-MSCs Overexpress TERT and MYOCD**

The percentage of YFP–TERT–transduced AT-MSCs was examined by using FACS. The percentage of TERT–YFP–expressing cells increased with the multiplicity of infection (Online Figure IB). MYOCD-V5 expression was analyzed by means of Western blot analysis. At 5 days post-transduction, we observed an increase in the expression of both MYOCD (Online Figure IC) and MYOCD-V5 (Online Figure ID) that correlated with the increased multiplicity of infection. Online Figure IE shows positive (heart) and negative (liver) controls for MYOCD.

**Overexpression of TERT and MYOCD Confers Increased Clonogenic Capacity to Murine AT-MSCs**

To examine the ex vivo proliferative potential of AT-MSCs from 1- and 12-month-old C57 or ApoE−/− mice, we performed in vitro clonogenic assays in methylcellulose. In these experiments, individual colonies are theoretically derived from a single MSC, and the size of the colony at a given time reflects the proliferative capacity of the starting cell. We found that the colony number and size of AT-MSCs were significantly lower in AT-MSCs from aged (12 months old) C57 and ApoE−/− mice than in those from young (1 month old) mice (n=5; P<0.05), regardless of whether the cells were transduced alone, suggesting that MYOCD overexpression did not interfere with TERT-mediated clonogenic activity. The results from these assays suggest that the increase in colony size and number that we observed after MSCs were transduced with TERT with or without MYOCD may have resulted from either faster proliferation or better survival in culture.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Number</th>
<th>Size*</th>
<th>Number</th>
<th>Size*</th>
<th>Number</th>
<th>Size*</th>
<th>Number</th>
<th>Size*</th>
<th>Number</th>
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<tr>
<td>C57 1 mo</td>
<td>5±2</td>
<td>1.50±0.43</td>
<td>4±1</td>
<td>1.25±0.49</td>
<td>5±3</td>
<td>1.67±0.53</td>
<td>11±5†</td>
<td>4.00±0.9‡</td>
<td>13±6†</td>
<td>3.80±1‡</td>
</tr>
<tr>
<td>12 mo</td>
<td>1±2‡</td>
<td>0.10±0.05‡</td>
<td>1±1†</td>
<td>0.25±0.08‡</td>
<td>2±1†</td>
<td>0.50±0.10‡</td>
<td>6±2†</td>
<td>3.50±1†</td>
<td>6±2†</td>
<td>3.0±0.80‡†</td>
</tr>
<tr>
<td>ApoE−/− 1 mo</td>
<td>6±2</td>
<td>0.50±0.08</td>
<td>5±1</td>
<td>0.60±0.04</td>
<td>6±4</td>
<td>0.80±0.06</td>
<td>12±4†</td>
<td>2.80±0.8‡†</td>
<td>15±7†</td>
<td>2.50±0.50‡†</td>
</tr>
<tr>
<td>12 mo</td>
<td>1±1†</td>
<td>0.08±0.00</td>
<td>1±1†</td>
<td>0.06±0.00</td>
<td>2±1†</td>
<td>0.09±0.03</td>
<td>4±2†</td>
<td>1.60±0.60‡†</td>
<td>5±2†</td>
<td>1.50±0.30‡†</td>
</tr>
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</table>

Values are presented as the mean±SD of counted colony-forming units (CFU)/10^6 cells or of colony size (diameter) (n=5 mice per group). ApoE−/− indicates apolipoprotein E deficient; AT-MSCs, adipose tissue–derived mesenchymal stromal cells; C57, C57/BL6; Mock, mock-transduced AT-MSCs; MYOCD, MYOCD transduced with pLenti-myocardin vector; MYOCD+TERT, transduced with pLenti-TERT and pLenti-MYOCD vectors; NT, nontransduced; and TERT, transduced with pLenti-telomerase reverse transcriptase (TERT) vector.

*Measured in mm.
†P<0.05 vs 1 mo.
‡P<0.05 vs mock-transduced AT-MSCs.
showed that MSCs overexpressing both TERT and MYOCD exhibited a highly proliferative phenotype, whereas the overexpression of MYOCD alone did not alter the growth rate of MSCs.

To examine the effect of TERT and MYOCD overexpression on MSC death, we performed flow cytometry analysis of SYTOX Red–stained BM-MSCs (from 12-month-old C57 mice) that were mock-transduced or transduced with TERT or MYOCD (Figure 1A). SYTOX Red stains dead and dying cells by permeating compromised cell membranes to stain nuclear chromatin. Twenty-one days after transduction, we observed a large decrease in cell death in MYOCD-overexpressing BM-MSCs compared with mock-transduced BM-MSCs (1.2±0.5% versus 7.7±0.9%; n=5; P<0.01). To a lesser extent, we observed a decrease in cell death in TERT-overexpressing BM-MSCs when compared with mock-transduced BM-MSCs (5.1±3.8% versus 7.7±0.9%; n=5; P<0.05; Figure 1A). Together, these data suggest that TERT and MYOCD overexpression prevents cytotoxic cell death.

To evaluate the effect of TERT and MYOCD overexpression on the resistance of MSCs to apoptosis and necrosis, we performed flow cytometry analysis of PI and Annexin V staining in Fas ligand–stimulated BM-MSCs from 12-month-old C57 mice 21 days after the transduction of MYOCD or TERT. Compared with mock-transduction, the overexpression of TERT or MYOCD in BM-MSCs conferred a greater resistance to Fas-induced and non–Fas-induced apoptosis (n=5; P<0.05) and decreased the percentage of total cell death (n=5; P<0.05; Figure 1B and 1C). More specifically, in response to Fas ligand stimulation, the fraction of Annexin V+/PI− cells (Q-II, early apoptosis) and Annexin V+/PI+ cells (Q-III, late apoptosis) was significantly lower in TERT-overexpressing cells (0.33±0.03 versus 0.46±0.09*; n=5; P<0.01) and MYOCD overexpressing cells (0.12±0.01 versus 0.22±0.05*; n=5; P<0.05) compared with mock-transduced cells (0.35±0.03 versus 0.45±0.03*; n=5; P<0.01). To a lesser extent, TERT, protects MSCs from apoptosis.

Overexpression of TERT and MYOCD Differentially Influences MSC Differentiation

To determine whether MSCs overexpressing TERT and MYOCD retained a normal differentiation response and whether the response was altered by age, we examined the mesenchymal (osteogenic and adipogenic) and myogenic differentiation potential of AT-MSCs overexpressing TERT, MYOCD, or both TERT and MYOCD.

We directed AT-MSCs toward osteogenic or adipogenic lineages and compared their differentiation efficiency by using histochemical staining. After 21 days of osteogenic differentiation and 14 days of adipogenic differentiation, Alizarin Red S (Figure 2) and Oil Red O (Figure 3) staining was significantly higher in AT-MSCs from 1-month-old C57 and ApoE−/− mice than in AT-MSCs from 12-month-old mice (n=5; P<0.01 versus 12-month-old mice), respectively, suggesting a higher osteogenic and adipogenic differentiation potential in cells from young mice than in cells from aged mice. When compared with mock-transduced AT-MSCs, AT-MSCs from 1- and 12-month-old C57 or ApoE−/− mice transduced with TERT had an elevated osteogenic differentiation potential (n=5; P<0.01; Figure 2) and a decreased adipogenic differentiation potential (n=5; P<0.01; Figure 3). Thus, the overexpression of TERT was associated with an inverse relationship between osteogenic and adipogenic differentiation. Furthermore, AT-MSCs from 1- and 12-month-old C57 or ApoE−/− mice overexpressing MYOCD also exhibited decreased adipogenic differentiation (n=5; P<0.05 versus mock-transduced), and when overexpressed with TERT, MYOCD potentiated the TERT-mediated reduction in adipogenic differentiation (n=5; P<0.01 versus TERT-transduced AT-MSCs; Figure 3). The overexpression of MYOCD with TERT did not alter TERT-mediated osteogenic differentiation (Figure 2).

To evaluate the myogenic differentiation potential of AT-MSCs, we compared the expression of the myogenic markers cardiac actin and smooth muscle α-actin in AT-MSCs, BM-MSCs, and human BM mesenchymal stem cells overexpressing TERT, MYOCD, or both TERT and MYOCD. Compared with mock-transduced AT-MSCs, AT-MSCs overexpressing TERT and MYOCD showed an increase in both smooth muscle α-actin and cardiac actin expression (n=5; P<0.05 and P<0.01 versus mock-transduced AT-MSCs,

**Table 2. Effect of TERT and MYOCD Transduction on Cell Proliferation in Murine AT-MSCs from Young (1 Month Old) and Aged (12 Months Old) C57/B6L and ApoE−/− Mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>NT</th>
<th>Mock</th>
<th>MYOCD</th>
<th>TERT</th>
<th>MYOCD+TERT</th>
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<tr>
<td>C57</td>
<td></td>
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<tr>
<td>1 mo</td>
<td>0.34±0.02</td>
<td>0.35±0.03</td>
<td>0.33±0.03</td>
<td>0.46±0.09*</td>
<td>0.45±0.03*</td>
</tr>
<tr>
<td>12 mo</td>
<td>0.15±0.05†</td>
<td>0.13±0.05†</td>
<td>0.12±0.01†</td>
<td>0.22±0.05†</td>
<td>0.28±0.10†</td>
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<td>ApoE−/−</td>
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<tr>
<td>1 mo</td>
<td>0.33±0.06</td>
<td>0.32±0.04</td>
<td>0.34±0.05</td>
<td>0.48±0.10*</td>
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</tr>
<tr>
<td>12 mo</td>
<td>0.10±0.01†</td>
<td>0.10±0.07†</td>
<td>0.09±0.01†</td>
<td>0.16±0.05†</td>
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</table>

Values are presented as the mean±SD of absorbance units (O.D.) at 450/595 nm, which represents the uptake of bromodeoxyuridine (BrdU) in cells (n=5 mice per group). ApoE−/− indicates apolipoprotein E deficient; AT-MSCs, adipose tissue–derived mesenchymal stromal cells; C57, C57/BL6; Mock, mock-transduced AT-MSCs; MYOCD, transduced with pLenti-myocardin vector; MYOCD+TERT, transduced with pLenti-TERT and pLenti-MYOCD vectors; NT, nontransduced AT-MSCs; and TERT, transduced with pLenti-telomerase reverse transcriptase (TERT) vector.

*P<0.05 vs mock-transduced AT-MSCs.
†P<0.01 vs 1 mo.
Figure 1. Total cell death and apoptosis in bone marrow mesenchymal stromal cells (BM-MSC) or adipose tissue-derived mesenchymal stromal cells (AT-MSCs) from aged C57/BL6 mice transduced with or without telomerase reverse transcriptase (TERT), myocardin (MYOCD), or both TERT and MYOCD. A, Representative images show flow cytometry analysis of SYTOX Red staining and yellow fluorescent protein (YFP-A) expression in mock-transduced BM-MSCs or BM-MSCs transduced with TERT or MYOCD (n=5 mice per group). SYTOX Red (APC-A) was detected as a measure of total cell death. MOI indicates multiplicity of infection; PE, phycoerythrin; and SSC, side scatter. B, Representative flow cytometry results of Annexin-fluorescein isothiocyanate (FITC) and propidium iodide (PI-A) staining in mock-transduced BM-MSCs and BM-MSCs transduced with TERT or MYOCD (n=3 mice per group) treated with or without FAS/CD95 ligand (500 ng/mL). Staining for Annexin V, propidium iodide, or both, shows that apoptosis is reduced in TERT- or MYOCD-overexpressing BM-MSCs. Results are from 3 independent experiments performed in triplicate. Quadrants are defined as follows: live (bottom left, Q-I), necrotic (top left, Q-IV), early apoptotic (bottom right, Q-II), late apoptotic (top right, Q-III). C, Flow cytometry quantification of total cell death, necrotic cell death, and apoptotic cell death in mock-transduced BM-MSCs and BM-MSCs transduced with TERT or MYOCD treated with or without Fas/CD95 ligand (500 ng/mL; n=3). D, Representative immunoblot analysis of Annexin V (Ann-V), caspase-3, and cleaved caspase-3 protein expression in mock-transduced AT-MSCs or AT-MSCs transduced with TERT, MYOCD, or both TERT and MYOCD (n=5 mice per group). The blots were stripped and reincubated with β-actin antibody. E, Densitometric analysis of the protein bands shown in (D). Results are representative of 3 different experiments of 5 animals each, and the data are presented as the mean±SD. *P<0.05 and **P<0.01 vs mock-transduced BM-MSCs or AT-MSCs.
young (1 month old) C57/BL6 (C57) and ApoE−/− mice during differentiation analysis, representative images show Alizarin Red S staining in AT-MSCs from aged (12 months old) and young (1 month old) C57/BL6 and ApoE−/− mice that were nontransduced (Nt), mock-transduced, or transduced with TERT, MYOCD, or both TERT and MYOCD (n=5 mice per each group). Magnification=10×. B, The degree of Alizarin Red S mineralization was quantified by normalizing absorbance (O.D.) at 450 nm to the relative number of viable cells. Graph represents combined data from 3 independent experiments of 5 samples each; results are presented as the mean±SD (n=5 mice per group). **P<0.01, ***P<0.001, and ****P<0.0001 versus mock-transduced AT-MSCs of 1-month-old mice; °°P<0.01, °°°P<0.001, and °°°°P<0.0001 versus 12-month-old mice; °°°°P<0.0001 versus mock-transduced GFP+ AT-MSCs, as indicated by the absence of cell infiltrates on hematoxylin and eosin–stained tissue sections.31,32 To evaluate the potential paracrine capacity of MSCs overexpressing TERT, MYOCD, or both TERT and MYOCD, we evaluated VEGF protein levels in AT-MSC extracts and cell supernatants by using an enzyme-linked immunosorbent assay. VEGF was more highly expressed in AT-MSC extracts from 1-month-old C57 and ApoE−/− mice than in those from 12-month-old-mice (n=5; P<0.05 versus 12-month-old mice; Table 3). Importantly, AT-MSCs from 1- and 12-month-old C57 and ApoE−/− mice overexpressing TERT had elevated paracrine activity when compared with that of mock-transduced AT-MSCs (n=5; P<0.05 versus mock-transduced AT-MSCs). MYOCD overexpression alone had no effect on VEGF expression, and the overexpression of MYOCD with TERT did not interfere with the TERT-mediated increase in VEGF expression. Similar qualitative findings were observed in cell supernatants (data not shown).

In Vivo Transplantation of GFP+ AT-MSCs Overexpressing TERT and MYOCD Improves Blood Flow in the Ischemic Hindlimb of ApoE−/− Mice

Given that AT-MSCs overexpressing TERT, MYOCD, or both TERT and MYOCD showed evidence of increased proliferative potential, decreased apoptosis, and increased VEGF production, we hypothesized that the overexpression of TERT and MYOCD would augment the blood flow recovery effects of AT-MSCs transplanted after hindlimb ischemia. To examine the physiological in vivo effect of TERT and MYOCD overexpression, mock-transduced GFP+ AT-MSCs, GFP+ AT-MSCs overexpressing TERT and MYOCD, or PBS was injected into the ischemic hindlimbs of ApoE−/− mice by means of multiple intramuscular injections. Blood flow was measured before femoral ligation, 1 day after ligation (preinjection), and 2 weeks after treatment. Representative laser Doppler images (Figure 4A) showed perfusion of the ischemic (right) legs versus the nonischemic contralateral limbs. Before femoral ligation, baseline blood flow was better in C57 mice than in ApoE−/− mice (Figure 4A, top). Fifteen days after the induction of unilateral ischemia, ApoE−/− mice treated with mock-transduced GFP+ AT-MSCs showed a moderate but significantly greater recovery of limb perfusion than did mice treated with PBS (n=5; P<0.05; Figure 4A and 4B). Furthermore, ApoE−/− mice treated with GFP+ AT-MSCs overexpressing TERT and MYOCD showed an even greater recovery of limb perfusion than did those treated with mock-transduced GFP+ AT-MSCs (n=5; P<0.05; Figure 4A and 4B). No evidence of neoplastic transformation or inflammation was observed at the injection site in mice treated with GFP+ AT-MSCs overexpressing TERT and MYOCD or mock-transduced GFP+ AT-MSCs, as indicated by the absence of cell infiltrates on hematoxylin and eosin–stained slides of ischemic leg muscle (Online Figure IV).
In Vivo Transplantation of GFP+ AT-MSCs Overexpressing TERT and MYOCD Improves Arteriogenesis in the Ischemic Hindlimb of ApoE−/− Mice

Because neovascularization is thought to be essential for maintaining perfusion recovery, we examined arteriogenesis in the ischemic hindlimbs of ApoE−/− mice after cell therapy. To identify arterioles and capillaries, we used antibodies against smooth muscle α-actin and von Willebrand factor to immunostain tissue sections of ischemic and contralateral nonischemic legs 21 days after treatment. Capillary and arteriole density were markedly increased in mice that received mock-transduced GFP+ AT-MSCs (n=5; P<0.05) when compared with those that received PBS (Figure 5A–5D; Online Table). Capillary and arteriole density were even further increased in mice that received GFP+ AT-MSCs overexpressing TERT and MYOCD when compared with those that received mock-transduced GFP+ AT-MSCs (n=5; P<0.05; Figure 5A–5D; Online Table).

In Vivo Transplantation of GFP+ AT-MSCs Overexpressing TERT and MYOCD Results in Cell Engraftment into Ischemic Tissues and Cellular Differentiation into Vascular Structures

To examine the engraftment and incorporation of transplanted GFP+ AT-MSCs into vascular structures, we histologically examined the long-term engraftment of cells on transverse sections of cell-treated legs. Cell retention 21 days after the transplantation of mock-transduced GFP+ AT-MSCs and GFP+ AT-MSCs overexpressing TERT and MYOCD is shown in Online Figure VA and VB, respectively. GFP+ cells were found in skeletal muscle in the areas of GFP+ AT-MSC injections, whereas no GFP+ cells were found in skeletal muscle that did not receive cell delivery (data not shown). At 21 days after transplantation, the number of GFP+ AT-MSCs overexpressing TERT and MYOCD was much higher than that of mock-transduced GFP+ AT-MSCs (420±120 cells versus 220±87 cells; P<0.05) and PBS-treated GFP+ AT-MSCs (420±120 versus 0±0; P<0.01), indicating an increase in cell engraftment or in vivo proliferation of GFP+ AT-MSCs overexpressing TERT and MYOCD.

Figure 3. Adipogenic differentiation in adipose tissue–derived mesenchymal stromal cells (AT-MSCs) from aged and young C57/BL6 and ApoE−/− mice transduced with or without telomerase reverse transcriptase (TERT), myocardin (MYOCD), or both TERT and MYOCD. A and B, Representative images showing Oil Red O staining of lipid accumulation in AT-MSCs that differentiated down an adipocyte lineage. AT-MSCs were from aged (12 months old) and young (1 month old) (A) C57/BL6 (C57) and (B) ApoE−/− mice that were nontransduced (Nt), mock-transduced, or transduced with TERT, MYOCD, or both TERT and MYOCD. A and B, Representative images showing Oil Red O staining of lipid accumulation in AT-MSCs that differentiated down an adipocyte lineage. AT-MSCs were from aged (12 months old) and young (1 month old) (A) C57/BL6 (C57) and (B) ApoE−/− mice that were nontransduced (Nt), mock-transduced, or transduced with TERT, MYOCD, or both TERT and MYOCD. C, The degree of adipocyte differentiation was quantified by normalizing the absorbance (O.D.) of Oil Red O at 490 nm to the relative number of viable cells. Graph represents combined data from 3 independent experiments; results are presented as the mean±SD. °°P<0.01, nontransduced, mock-transduced, MYOCD-transduced, TERT-transduced, and TERT+MYOCD-transduced AT-MSCs of 12-month-old mice vs the corresponding treatment groups of 1-month-old mice; **P<0.01, TERT+MYOCD-transduced and TERT-transduced AT-MSCs of 12- or 1-month-old mice vs mock-transduced AT-MSCs of 12- or 1-month-old mice; *P<0.05, MYOCD-transduced AT-MSCs of 12- or 1-month-old mice vs mock-transduced AT-MSCs of 12- or 1-month-old mice.
To further determine whether TERT and MYOCD transduction would affect cell proliferation of the parenchyma surrounding the injection site, we analyzed the percentage of Ki-67–positive cells in the ischemic leg muscle of cell-treated ApoE−/− mice. At 21 days after transplantation, ApoE−/− mice that received GFP+ AT-MSCs overexpressing TERT and MYOCD had a significantly higher percentage of Ki-67–positive cells than did those that received mock-transduced GFP+ AT-MSCs (n=5; P<0.01; Online Figure VIA and VIB), indicating increased cell proliferation in the parenchyma surrounding the application site of GFP+ AT-MSCs overexpressing TERT and MYOCD.

To determine whether transplanted cells integrated into the vasculature directly or had a more indirect perivascular effect, we performed morphometric analysis of the ischemic leg tissues of cell-treated ApoE−/− mice 21 days after transplantation. Using a CRi Nuance multispectral imaging system, we quantified the colocalization of GFP expression with nuclei (DAPI) and smooth muscle α-actin. Multispectral imaging of transverse leg sections immunostained for smooth muscle α-actin (DAPI) and smooth muscle-α-actin revealed that GFP expression colocalized with smooth muscle α-actin and DAPI staining in vascular structures (Online Figure VII), suggesting the incorporation of mock-transduced GFP+ AT-MSCs (n=5; P<0.01; Online Figure VIA and VIB), indicating increased cell proliferation in the parenchyma surrounding the application site of GFP+ AT-MSCs overexpressing TERT and MYOCD. Colocalization of GFP with DAPI and smooth muscle α-actin was much higher in the ischemic leg muscles of ApoE−/− mice transplanted with GFP+ AT-MSCs overexpressing TERT and MYOCD than in those transplanted with mock-transduced GFP+ AT-MSCs.

### Discussion

In our study, MSCs lentivirally transduced to overexpress TERT and MYOCD showed evidence of increased proliferation, decreased cell death, increased myogenic differentiation potential, and increased VEGF production when compared with mock-transduced MSCs. Furthermore, in an ApoE−/− model of hindlimb ischemia, the transplantation of aged MSCs overexpressing TERT and MYOCD improved limb perfusion and increased arteriogenesis. In ischemic tissues of ApoE−/− mice, we observed evidence of cell engraftment and differentiation into vascular structures. Our findings suggest that MSCs that are transduced and rejuvenated with TERT and MYOCD may have therapeutic applications for use in treating patients with vascular disease, particularly patients with age-related vascular disease.

Previous reports have shown that the maintenance of telomerase activity during the differentiation of embryonic stem cells enhances proliferation, provides resistance to apoptosis, and improves differentiation toward hematopoietic lineages by expansion of the progenitor population. In telomerase-deficient mice, which exhibit severe tissue degeneration and significant progeroid phenotypes, the recovery of telomerase and telomere function results in the restoration of proliferation in quiescent cultures and eliminates degenerative phenotypes in multiple organs, including the testes, brain, spleen, and intestines. In aged MSCs, TERT promotes cell growth and self-renewal by disrupting p53 activity and enhances cell migration through cortactin deacetylation. In addition, conditional ablation of the MYOCD gene in cardiomyocytes results in increased apoptosis and rapid progression of dilated cardiomyopathy and heart failure. In agreement with these previous findings, our results indicate that aged MSCs overexpressing TERT and MYOCD exhibit increased proliferation, self-renewal, and differentiation potential. We also found that aged MSCs transduced with MYOCD—and, to a lesser extent, MSCs transduced with TERT—showed greater resistance to apoptosis than did mock-transduced MSCs. These findings are partially in agreement with those of Chen et al., who were the first to show that smooth muscle cells overexpressing MYOCD have a low growth potential, and Tang et al., who showed that MYOCD functions as an anti proliferative factor in smooth muscle cells by interfering with nuclear factor-κB–dependent cell cycle regulation without inducing apoptosis. In our study, MSCs overexpressing TERT proliferated more rapidly than mock-transduced MSCs, whereas MSCs overexpressing MYOCD did not. Similar to MSCs overexpressing TERT, MSCs overexpressing both TERT and MYOCD exhibited a highly proliferative phenotype, which indicates that MYOCD does not interfere with TERT-mediated effects on proliferation. The slightly different effects of MYOCD on MSCs and on mature smooth muscle cells may reflect the...
ApoE−/− ischemia was induced in 12-month-old mice transduced with or without telomerase reverse transcriptase (TERT) and myocardin (MYOCD). Hindlimb mice) transduced with or without telomerase reverse transcriptase (TERT) and myocardin (MYOCD).

Blood flow analysis in ApoE−/− mice with hindlimb ischemia after the intramuscular injection of adipose tissue–derived mesenchymal stromal cells (AT-MSCs; from 12-month-old green fluorescent protein [GFP]–expressing mice) transduced with or without telomerase reverse transcriptase (TERT) and myocardin (MYOCD). Hindlimb ischemia was induced in 12-month-old ApoE−/− mice (ligation; n=5). The nonligated contralateral limb was used as a control (no ligation). Nonligated 12-month-old C57BL6 (C57) mice (n=5) were also used as a control. Mice received an injection of phosphate-buffered saline (PBS) or AT-MSCs from 12-month-old mice expressing GFP (GFP+) that were either mock-transduced or transduced with TERT and MYOCD (TERT+MYOCD). A. A laser Doppler perfusion imaging system was used to analyze blood flow 1 day before ligation (baseline), 1 day after ligation, and 2 weeks after the injection of PBS or transduced GFP+ AT-MSCs (ie, 15 days after ligation). Insets in A, left, the site of mock-transduced GFP+ AT-MSC injections; right, the site of TERT and MYOCD–transduced GFP+ AT-MSC injections. B. Blood flow recovery, expressed as the ratio of the right (ischemic) leg to the left (nonischemic) leg. Graphs represent combined data

We also observed that the transplantation of AT-MSCs overexpressing TERT and MYOCD resulted in cell engraftment into ischemic tissues and cellular differentiation or integration.

Figure 4. Blood flow analysis in ApoE−/− mice with hindlimb ischemia after the intramuscular injection of adipose tissue–derived mesenchymal stromal cells (AT-MSCs; from 12-month-old green fluorescent protein [GFP]–expressing mice) transduced with or without telomerase reverse transcriptase (TERT) and myocardin (MYOCD). Hindlimb ischemia was induced in 12-month-old ApoE−/− mice (ligation; n=5). The nonligated contralateral limb was used as a control (no ligation). Nonligated 12-month-old C57BL6 (C57) mice (n=5) were also used as a control. Mice received an injection of phosphate-buffered saline (PBS) or AT-MSCs from 12-month-old mice expressing GFP (GFP+) that were either mock-transduced or transduced with TERT and MYOCD (TERT+MYOCD). A. A laser Doppler perfusion imaging system was used to analyze blood flow 1 day before ligation (baseline), 1 day after ligation, and 2 weeks after the injection of PBS or transduced GFP+ AT-MSCs (ie, 15 days after ligation). Insets in A, left, the site of mock-transduced GFP+ AT-MSC injections; right, the site of TERT and MYOCD–transduced GFP+ AT-MSC injections. B. Blood flow recovery, expressed as the ratio of the right (ischemic) leg to the left (nonischemic) leg. Graphs represent combined data

activation of different signal transduction pathways in these different cell types.

A large body of evidence has indicated that telomerase has roles in cellular processes independent of its role in telomere maintenance, including the activation of VEGF expression and the induction of angiogenic properties of endothelial cells and their precursors. In agreement with the results of other studies showing that TERT increases angiogenic properties, we found that TERT overexpression induced VEGF expression in MSCs. Interestingly, it was recently shown that the response of VEGF to TERT induction is inhibited in senescent endothelial cells. Remarkably, in our experiments, TERT overexpression restored VEGF production in aged MSCs, and MYOCD did not interfere with the TERT-mediated increase in VEGF expression. Therefore, TERT may control a proangiogenic molecular network by increasing VEGF production. Given that the TERT-dependent increase in VEGF function may be compromised in aging, the delivery of TERT and MYOCD genes into MSCs may restore the proangiogenic paracrine activity of these cells. Our results provide evidence of a link between telomerase expression and the angiogenic effects of transplanted MSCs.

In this study, the process of arteriogenesis involves the proliferation, survival, and potential myogenic differentiation of transplanted MSCs. The data from this study support our previous finding that TERT may have a role in determining the myogenic stemness of MSCs, that is, maintaining MSCs in an intermediate biological window in which an undifferentiated, uncommitted stem cell evolves toward myogenic commitment while maintaining potency for proliferation. Furthermore, our data reinforce the concept that MYOCD and TERT work synergistically to promote promyogenic gene expression and maintain the growth capacity of MSCs. The differences in stemness and survival that we observed between mock-transduced AT-MSCs and AT-MSCs overexpressing MYOCD and TERT are reflected in the angiogenic potential of each cell type. AT-MSCs overexpressing MYOCD and TERT showed better capillary and arteriole formation than did mock-transduced AT-MSCs after transplantation into ischemic tissues. This proangiogenic property of AT-MSCs overexpressing MYOCD and TERT may reflect either a decrease in the loss of vascular cells attributable to the antiapoptotic/necrotic properties of these cells, or a direct replenishment of smooth muscle cells in ischemic muscles after the expansion of the myogenic progenitor population. Alternatively, the increased vascular density observed in ischemic tissues treated with AT-MSCs overexpressing MYOCD and TERT may reflect a response to the release of proangiogenic growth factors that was shown in our study to be potentiated by the overexpression of TERT and MYOCD in MSCs in vitro.

We also observed that the transplantation of AT-MSCs overexpressing TERT and MYOCD resulted in cell engraftment into ischemic tissues and cellular differentiation or integration.

Figure 4 (Continued). from 3 independent experiments (n=5 mice per group); results are presented as the mean±SD. **P<0.01 and *P<0.05 vs PBS-treated ApoE−/− mice without ligation; °P<0.05 vs PBS-treated ApoE−/− mice with ligation; #P<0.05 vs ApoE−/− mice with ligation and treated with mock-transduced AT-MSCs.
into vascular structures, as shown by the proliferation of these cells and their colocalization with nuclei and smooth muscle cells. However, it is not clear whether this colocalization and concomitant increase in arteriogenesis resulted from the fusion of transplanted cells with native vasculature (and possibly vascular rescue attributable to decreased cell death), the directed differentiation of transplanted cells, or the paracrine-mediated secretion of proangiogenic growth factors by the transplanted cells in vivo. Distinguishing among these possibilities requires further investigation.

Peripheral artery disease resulting from atherosclerosis produces chronic limb ischemia. Similarly, when ApoE<sup>−/−</sup> mice (8–12 months old) are fed a normal chow diet, they develop spontaneous atherosclerosis that results in the narrowing of the vessel lumen, which leads to the progressive restriction of blood flow at multiple arterial branches, including the hindlimb vessels. Thus, for our study, we used 12-month-old ApoE<sup>−/−</sup> mice as cell therapy recipients because they develop chronic atherosclerosis similar to the atherosclerotic lesions observed in humans. In agreement with previous reports, we found that baseline blood flow in the nonischemic limb was better in C57 mice than in ApoE<sup>−/−</sup> mice.

Figure 5. Arteriogenesis in aged ApoE<sup>−/−</sup> mice with hindlimb ischemia after the intramuscular injection of aged adipose tissue–derived mesenchymal stromal cells (AT-MSCs) transduced with or without telomerase reverse transcriptase (TERT) and myocardin (MYOCD). Histological analysis of arteriogenesis is shown in the ischemic hindlimb of ApoE<sup>−/−</sup> mice that received injections of phosphate-buffered saline (PBS) or AT-MSCs from 12-month-old mice expressing green fluorescent protein (GFP) that were mock-transduced or transduced with TERT and MYOCD (n=5 mice per group). The nonischemic contralateral limb is shown as a control. Representative photomicrographs are shown of capillaries and arterioles (A and B) and arterioles (C and D) in tissue sections taken from the adductor and semimembranous muscles at 1 day after ligation and at 21 days after treatment, followed by staining with alkaline phosphatase–conjugated secondary antibodies against von Willebrand factor and smooth muscle α-actin (SM α-actin), respectively. The extent of arteriogenesis was determined by measuring arteriole and capillary density in light microscopic sections. Murine tonsils were used as a positive control for the staining of capillaries and arterioles. Scale bar=50 μm.
growth in culture or tumorigenicity in mice after transplantation, we observed no such neoplasticity. TERT overexpression by lentiviral transduction was limited to <4 weeks and did not bring about the immortalization of AT-MSCs. Because of the transient (and not stable) overexpression of TERT and MYOCD, overexpression beyond 4 weeks would be expected to be limited. To examine the time course of stability of TERT and MYOCD overexpression, the transduction would need to be done in a stable manner and the outcome followed over time. Although the fundamental mechanisms underlying the senescence of mammalian cells (and the senescence of the vasculature) remain to be elucidated, our findings indicate that impairment of the vascular response in older individuals may be partially restored by the transplantation of AT-MSCs rejuvenated in vitro by the delivery of TERT and MYOCD genes.

In summary, TERT and MYOCD gene transfer may rejuvenate and restore the myogenic development of aged MSCs derived from adult AT. The interaction between TERT and MYOCD in myogenic MSCs may be important in the timing of myogenesis and in the proliferation and differentiation of MSCs. The concept that MSCs can be rejuvenated to exhibit a delay in senescence and enhanced regenerative properties has therapeutic implications for vascular disorders, including myocardial ischemia, peripheral artery disease, and critical limb ischemia. In these disorders, the viability of MSCs and fully differentiated endothelial and smooth muscle cells is reduced by a variety of individual and environmental stress factors. MSCs transduced to overexpress TERT and MYOCD may have therapeutic applications for use in the repair and regeneration of peripheral vasculature and its coronary counterpart.

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Disclosures

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References


31. Gneccchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noisieux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005;11:367–368.


Novelty and Significance

What Is Known?

- The number and function of stem cells decline with age, reducing the ability of stem cells to contribute to endogenous repair processes.
- The catalytic subunit of telomerase—telomerase reverse transcriptase (TERT)—and the transcription factor and antiapoptotic protein myocardin (MYOCD) are highly expressed in a subpopulation of mesenchymal cells (MSCs) derived from adipose tissue and may act together to enhance myogenic cardiovascular development.
- An appropriate therapy for age-related vascular disease may be to restore stem cell function by rejuvenating the existing stem cells that can in turn supply the ischemic tissue with new vessels.

What New Information Does This Article Contribute?

- The delivery of the TERT and MYOCD genes restored MSCs from aged mice by decreasing cell apoptosis and increasing cell survival, proliferation, and smooth muscle myogenic differentiation in vitro.
- MSCs rejuvenated by TERT and MYOCD overexpression provided therapeutic benefits in a mouse model of hindlimb ischemia by increasing blood flow and arteriogenesis through paracrine mechanisms and differentiation into vascular structures.
- Autologous MSCs overexpressing TERT and MYOCD could be a potential therapy in patients with vascular disease, particularly patients with age-related vascular disease.

The repair capacity of stem cells is reduced with age and may be improved by genetically reprogramming older stem cells to exhibit delayed senescence and enhanced regenerative properties. Previous studies have shown that TERT and MYOCD could act together to enhance myogenic cardiovascular development. Thus, we examined whether delivery of the TERT and MYOCD genes restores the myogenic function of aged MSCs. We found that MSCs lentivirally transduced to overexpress TERT and MYOCD show evidence of increased proliferation, decreased cell death, increased myogenic differentiation potential, and increased VEGF production when compared with mock-transduced MSCs. Furthermore, in a mouse model of hindlimb ischemia, transplantation of aged MSCs overexpressing TERT and MYOCD improved blood flow and increased arteriogenesis. We also observed evidence of cell engraftment and differentiation into vascular structures. These results suggest that delivery of TERT and MYOCD genes into MSCs may be a novel strategy for reducing stem cell senescence and enhancing the host response to ischemia in older patients. Importantly, MSCs rejuvenated by TERT and MYOCD overexpression may provide a plentiful source of myogenic cells for therapeutic use in heart and vessel regeneration.
Transplantation of Mesenchymal Cells Rejuvenated by the Overexpression of Telomerase and Myocardin Promotes Revascularization and Tissue Repair in a Murine Model of Hindlimb Ischemia

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SUPPLEMENTAL MATERIAL

Transplantation of Mesenchymal Cells Rejuvenated by the Overexpression of Telomerase and Myocardin Promotes Revascularization and Tissue Repair in a Murine Model of Hindlimb Ischemia

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Heart Failure Research (R.M., Y-J.G., H.S., J.T.W.), Regenerative Medicine Research (D.T.), and the Stem Cell Center (E.P., J.T.W.), Texas Heart Institute at St. Luke’s Episcopal Hospital, Houston, Texas; Department of Internal Medicine, Cardiology, The University of Texas Health Science Center at Houston, Houston, Texas (Y-J.G.); and Institute of Cardiology, Department of Neuroscience and Imaging, “G. d’Annunzio” University, Chieti, Italy (R.M., R.D.)
Detailed Methods

Isolation of Mesenchymal Stromal Cells (MSCs) and Cell Culture

Three strains of mice were purchased from the Jackson Laboratory (Sacramento, CA) for these experiments: male C57/BL6 (C57) mice (2 ages, one month old and 12 months old), apolipoprotein-deficient (ApoE−/−) mice (2 ages, one month old and 12 months old), and green fluorescent protein (GFP)-transgenic mice (12 months old). The GFP-transgenic mice were only used as a source of cells for the injections administered during the in vivo experiments. To obtain adipose tissue–derived mesenchymal stromal cells (AT-MSCs), the mice were anesthetized by isoflurane inhalation (2–5% isoflurane in oxygen) and then euthanized. AT-MSCs were isolated from the peri-epididymal visceral adipose tissue of each mouse according to a modified version of a protocol originally described by Zuk and colleagues.1 Briefly, adipose tissue was minced mechanically and digested with collagenase. After adipocyte removal, the vascular stromal fraction was plated at a density of 1,000 cells/cm² in Iscove’s Modified Dulbecco’s Medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin sulfate (100 μg/mL), nonessential amino acids (0.1 mmol/L), 2-mercaptoethanol (1 mmol/L), and 20% fetal bovine serum (FBS). After 24 hours, the AT-MSCs were selected on the basis of their plastic adherence properties, and the nonadherent cells were removed. Before transduction, the AT-MSCs were cultured in 100-mm dishes at high density for up to 3 passages. The majority of adherent cells were negative for CD31. At higher passages in culture, a small fraction of the adherent cells expressed CD34. Passage 3 AT-MSCs were characterized for their expression of markers for mesenchymal stem cells, endothelial cells, fibroblasts, smooth muscle cells, and monocytes/macrophages as described below.

Parallel experiments were performed with murine bone marrow-derived mesenchymal stromal cells (BM-MSCs). These were obtained by flushing the tibias and femurs of male C57 mice (age 12 months). Then, the vascular stromal fraction was plated at a density of 1,000 cells/cm² in Iscove’s Modified Dulbecco’s Medium (GIBCO, Invitrogen) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin sulfate (100
μg/mL), nonessential amino acids (0.1 mmol/L), and 20% FBS. After 24 hours, the BM-MSCs were selected on the basis of their plastic adherence properties, and the nonadherent cells were removed. The BM-MSCs at passage 3 were used for lentiviral transduction. Human bone marrow mesenchymal stem cells at passage 3 were used for control experiments (Lonza, Atlanta, GA).

**Characterization of Murine AT-MSCs by Using Flow Cytometry**

For flow cytometry analyses of endothelial progenitor cell markers, a total of $1 \times 10^6$ AT-MSCs were incubated for 30 min at 4°C with 10 μL of the following fluorescence-labeled monoclonal antibodies or their respective isotype control: (1) fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD34 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany); (2) phycoerythrin (PE)-conjugated mouse anti-CD133 antibody (Miltenyi Biotec); (3) pan-CD45 peridinin-chlorophyll-protein (PerCP)-conjugated antibody, which detects all isoforms and glycoforms of CD45 (BD Biosciences, San Jose, CA). For flow cytometry analyses of mesenchymal stem cell and monocyte/macrophage markers, cells were incubated with the following antibodies: (1) purified anti-mouse CD105 (endoglin) monoclonal antibody (Becton, Dickinson and Company, San Diego, CA), which specifically cross-reacts with (or is cross-specific for) the analogous rat antigen; (2) purified anti-mouse CD44 monoclonal antibody (Cedarlane Laboratories, Burlington, ON, Canada), which is cross-specific for the analogous rat antigen; (3) FITC-conjugated anti-human CD29 (ImmunoTools, Friesoythe, Germany); (4) FITC-conjugated anti-rat CD71 (ImmunoTools); (5) FITC-conjugated anti-human CD29 (ImmunoTools); (6) FITC-conjugated anti-rat CD106 (Santa Cruz Biotechnology, Santa Cruz, CA); and (7) FITC-conjugated anti-rat CD14 (ImmunoTools). For flow cytometry analysis of pericytes and smooth muscle cell lineages, cells were incubated with the following antibodies: (1) FITC-conjugated anti–human desmin (Sigma-Aldrich); and 2) purified human anti–smooth muscle cell actin (Sigma-Aldrich). Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). For
each sample, 30,000 events were acquired and analyzed with the CellQuest software (BD Biosciences).

**Cloning of Telomerase Reverse Transcriptase (TERT) and Myocardin (MYOCD)**

**Lentiviral Expression Plasmids**

Total RNA was isolated by using a Qiagen RNA isolation kit and was reverse transcribed directly into single-stranded cDNA by using the SuperScript™ III Platinum® One-Step RT-PCR System (Invitrogen) according to the manufacturer's protocol. Full-length cDNAs for human TERT (3.6 kb) and human MYOCD isoform 1 (3.1 kb) were amplified by means of PCR and subcloned into the pLenti-TOPO cloning vector (Invitrogen). The primers used for PCR amplification of murine TERT were 5’-CACCATGACCCGCGCTCCT-3’ (forward) and 5’-CGCCCAGTCCAAAATGGTCTG-3’ (reverse); the primers used for the PCR amplification of murine MYOCD were 5’-CACCATGACACTCCTGGGGTCTGAAC-3’ (forward) and 5’GTCCCACTGCTGTAAGTGGAGATCCAT-3’ (reverse). The full-length TERT and MYOCD cDNA sequences, as well as the constructs, were confirmed by using restriction enzyme digestion analysis and DNA sequencing.

**Lentiviral Production**

For lentiviral production, all cell culture procedures were performed under biosafety level 2 conditions, according to the experimental procedures previously described. The genome of the HIV-1-based lentivirus consists of several plasmids: the plasmid of interest and a mixture of 3 packaging plasmids (pLP1, pLP2, and pLP/VSVG), which supply the helper functions and the structural and replication proteins required to produce the lentivirus. The plasmids of interest encode the yellow fluorescent protein (YFP)-TERT or V5-MYOCD fusion proteins under the control of a cytomegalovirus promoter (CMV). To enhance the level of gene expression, we used a lentiviral vector that contained a spleen-focus forming virus (SFFV)-LTR promoter and a woodchuck hepatitis post-transcriptional regulatory element (WPRE) (Online Supplemental Figure IA). To obtain lentiviral particles, 293FT cells were plated 24 hours before transfection in 20-mm dishes in growth medium (10% high-glucose Dulbecco's Modified Eagle Medium [DMEM]) that did not contain antibiotics; the cells were subconfluent.
Forty micrograms of plasmid DNA was used for the transfection. To achieve precipitation, the plasmids were diluted with 100 µL of Opti-MEM and 120 µL of Lipofectamine 2000 to a final volume of 220 µL and incubated at room temperature for 20 minutes. The DNA / Lipofectamine 2000 complex was added dropwise to culture dishes and then incubated at 37°C in a 5% CO2 incubator overnight. After 24 hours, the medium was replaced with 20 mL of 10% high-glucose DMEM; this conditioned medium was collected after an additional 48 hours of incubation and filtered through a 0.45 µm cellulose acetate filter. To concentrate the pLenti-CMV-YFP/TERT and pLenti-CMV-V5/MYOCD vectors, 1 mL of medium containing the lentiviral vector was transferred to a 1.7-mL microcentrifuge tube and spun in a bench microcentrifuge at 18,000 g for 1 h at 4°C. The supernatant was then carefully removed from the tube. The pellet was gently resuspended into 100 µL of DMEM. The multiplicity of infection (MOI) was determined in 293T cells, AT-MSCs, and BM-MSCs by using serial dilutions of the vector stocks. AT-MSCs (2 x 10⁵ cells/well) were exposed to serial dilutions of the following vectors for 5 days: pLenti-CMV-V5 (mock), pLenti-CMV-MYOCD-V5, pLenti-CMV-YFP (mock), or pLenti-CMV-TERT-YFP; the serial dilutions corresponded with MOIs ranging from 1 to 40. Cells were infected with 50 µL (MOI = 1), 100 µL (MOI = 10), 200 µL (MOI = 20), or 400 µL (MOI = 40) of the concentrated vector. All of the in vivo and in vitro assays were performed at the infection level capable of producing the maximum efficiency of transduction while producing no cytotoxic effects.

Cytotoxicity Testing and Annexin V and Propidium Iodide Viability Assays

A live/dead viability/cytotoxicity kit containing SYTOX Red (Invitrogen) was used to measure the cytotoxicity of lentiviral transduction. Aliquots of 100 µl of murine wild-type or lentivirus-transduced BM-MSCs (mock or TERT/MYOCD–transduced cells) containing 1 x 10⁶ cells/mL in PBS were added to 50 µl of 0.5 µM SYTOX Red and incubated for 10 min at room temperature. Cells were washed and analyzed by using a BD FACSCanto II flow cytometer (BD Biosciences) equipped with a 633-nm laser (for excitation of SYTOX® Red, 640 nm). Data were analyzed by using FACS Diva software (BD Biosciences), and the
percentage of dead cells was represented by the percentage of SYTOX Red–positive cells. For the Annexin V/propidium iodide viability assays, 1 x 10⁶ murine wild-type or lentivirus-transduced BM-MSCs (mock or TERT/MYOCD–transduced cells) were plated in 60-mm culture dishes under serum-starved culture conditions (Dulbecco’s Modified Eagle Medium and 2% fetal calf serum) and treated overnight with Fas/CD95 (500 ng/mL). The Annexin V-Fluorescein Isothiocyanate (FITC) Kit from Pharmingen (Franklin Lakes, NJ) was used to measure cell viability. Briefly, cells were washed with cold PBS and resuspended with binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mmol/L CaCl₂). Annexin V and propidium iodide were added to the cell preparations and incubated for 25 min in the dark. A 1x Binding Buffer (400 μL; from 10x Binding Buffer with the following composition: 0.1 M HEPES [pH 7.4], 1.4 M NaCl, 25 mM CaCl₂; not provided with the kit) was then added to each tube. Samples were analyzed by using a BD FACSCanto II flow cytometer equipped with a 488-nm laser (for excitation of Annexin V-FITC, 488 nm and for propidium iodide, 535 nm). Data were analyzed by using FACS Diva software (BD Biosciences). Viable cells (those double negative for Annexin V-FITC and propidium iodide; ie, Annexin V-FITC⁻/PI⁻) are displayed in the lower left quadrant (Q-I); early apoptotic cells (Annexin V-FITC single positive; ie, Annexin V-FITC⁺/PI⁻) are in the lower right quadrant (Q-II); late apoptotic cells (double positive for Annexin V-FITC and PI; ie, Annexin V-FITC⁺/PI⁺) are in the upper right quadrant (Q-III); and necrotic cells (PI single positive; ie, Annexin V-FITC⁻/PI⁺) are in the upper left quadrant (Q-IV) of the data sets. Therefore, the percentage of total cell death was defined by the sum of all apoptotic and necrotic cells (Q-II + Q-III + Q-IV).

Osteogenic, Adipogenic, and Myogenic Differentiation Assays

For osteogenic differentiation analysis, we examined Alizarin Red S staining in nontransduced, mock-transduced, and MYOCD– and/or TERT-transduced AT-MSCs from 1- and 12-month-old C57 and ApoE⁻/⁻ mice. Cells were evaluated in duplicate. AT-MSCs were plated on 6-well plates at a density of 5 x 10³ cells/cm² in osteogenic differentiation medium (STEMPRO Osteogenesis Differentiation Kit, Invitrogen). At 21 days, cells were fixed in 4% paraformaldehyde and stained for 3 min with 2% Alizarin Red S solution (Sigma Aldrich, St.
Louis, MO). To quantify mineralization, Alizarin Red S precipitate was extracted by using a 10% acetic acid/20% methanol solution for 45 min. The extracted stain was then transferred to a 96-well plate, and the absorbance was measured at 450 nm by using a SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

For adipogenic differentiation analysis, we examined Oil Red O staining in nontransduced, mock-transduced, and MYOCD- and/or TERT-transduced AT-MSCs from 1- and 12-month-old C57 and ApoE−/− mice. Cells were evaluated in duplicate. Cells were plated on 6-well plates at a density of 1 x 10^4 cells/cm² in adipogenic differentiation medium (STEMPRO Adipogenesis Differentiation Kit, Invitrogen). After 14 days, cells were washed with PBS, fixed in a 10% solution of formaldehyde (Sigma Aldrich) for 1 h, washed with 60% isopropanol (Sigma), and stained with an Oil Red O solution (stock solution from Sigma Aldrich, diluted in 60% isopropanol) for 10 min. Cells were washed with tap water and destained in 100% isopropanol for 15 min. Images were collected by using an Olympus (Tokyo, Japan) microscope. Stained oil droplets were dissolved in isopropanol, and the amount was quantified by measuring the absorbance at 490 nm with a spectrophotometer.

For myogenic differentiation analysis, nontransduced, mock-transduced, and MYOCD- and/or TERT-transduced AT-MSCs and BM-MSCs from 12-month-old C57 mice and human mesenchymal stem cells were plated in 100-mm culture dishes at a density of 1,000 cells/cm² in high-density/high-serum culture in Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with l-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), 0.1 mM nonessential amino acids, 10⁻⁴ mol/l 2-mercaptoethanol, and 20% fetal bovine serum, as previously reported. After 15 days, cells were washed with PBS and detached by trypsinization. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Sigma Aldrich), and total protein was analyzed by means of immunoblotting for the myogenic markers cardiac actin and/or smooth muscle α-actin.

Unilateral Hind Limb Ischemia

By 8 months of age, ApoE−/− mice develop atherosclerotic vascular lesions in peripheral vessels when fed regular chow. Therefore, ApoE−/− mice (12 months old, 25-30 g) were
used as a model for testing the effectiveness of the AT-MSC treatments. The ApoE−/− mice were anesthetized by isoflurane inhalation (2–5% isoflurane in oxygen), and hindlimb ischemia was created by unilateral surgical ligation by using 2 adjacent sutures to interrupt the proximal left femoral artery and vein; the contralateral limb was used as a control. These mice were randomly assigned to different treatment groups (N=5 for each group): (a) allogeneic mock-transduced GFP+ AT-MSCs; (b) allogeneic GFP+ AT-MSCs transduced with TERT and MYOCD; or (c) phosphate buffered saline (PBS), as a noncellular control. One day after femoral ligation, the mice received 5 intramuscular injections (3 injections in the adductor muscle and 2 in the semimembranosus muscle in the ischemic leg) that contained PBS (500 µl) or a single dose (3 x 10^6 cells/500 µl) of lentiviral-transduced GFP+ AT-MSCs. The nonischemic legs received 5 intramuscular injections (3 injections in the adductor muscle and 2 in the semimembranosus muscle in the nonischemic leg) of PBS (500 µl). The AT-MSCs had been harvested from adult GFP-transgenic mice (12 months old) according to the protocol previously described. Using a Laser Doppler Perfusion Imager System (PIM II, Perimed), blood flow was measured in anesthetized animals one day before ligation (baseline), one day after ligation, and 2 weeks after the injections. Blood flow was also measured in non-ligated 12 month-old C57 mice (n=3). The mice were then euthanized, and tissues were harvested for western blot analysis and immunohistochemistry. The ischemic-to-nonischemic foot blood flow ratio was calculated as an index of blood flow recovery.

**Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Murine Vascular Endothelial Growth Factor (VEGF)**

Supernatants were collected from nontransduced, mock-transduced, and MYOCD- and/or TERT-transduced AT-MSCs from 1- and 12-month-old C57 and ApoE−/− mice 48 hours after the final change of fresh medium (50% DMEM, 50% F12, and 2% FBS). The concentration of murine VEGF in the cellular extracts and supernatants was determined by ELISA (Quantikine Mouse VEGF Immunoassay; R&D System, Minneapolis, MN) according to the manufacturer's instructions. A standard curve was prepared from 9 VEGF dilutions. The lower limit of sensitivity was found to be 8.4 pg/mL for the VEGF ELISA kit. The overall inter-
assay and intra-assay coefficients of variation were each <10%. The total cellular protein
congcentration in each well was measured with the bicinchoninic acid (BCA) assay (Pierce,
Rockford, IL). The VEGF content was normalized to the cell protein values.

**Histologic Evaluation of Capillary and Arteriolar Density and Cell Proliferation**

On day 21 after the injection of PBS or cells, the ischemic and nonischemic limbs of each
12-month-old ApoE<sup>−/−</sup> mouse were removed. Each hindlimb was transversely cut into 5 equal
sections (proximal to distal) and embedded in 5 separate paraffin blocks. Paraffin-embedded
limb sections were deparaffinized and rehydrated. We examined the effects of mock-
transduced GFP<sup>+</sup> AT-MSCs and GFP<sup>+</sup> AT-MSCs transduced with TERT and MYOCD on the
microvascular vessel density of capillaries and arterioles by performing
immunohistochemical analysis of von Willebrand factor and smooth muscle α-actin in 5 μm-
thick paraffin-embedded tissue sections of adductor and semimembranous muscles.

Sections were stained with a monoclonal antibody against smooth muscle α-actin (1:50
dilution; Sigma Aldrich) or a monoclonal antibody against von Willebrand factor (1:100
dilution, Sigma Aldrich). Vessels that stained positive only for von Willebrand factor were
identified as capillaries and arterioles, and vessels that stained positive for von Willebrand
factor and smooth muscle α-actin were identified as arterioles. Muscle sections were
counterstained with hematoxylin and eosin for structural analysis and to identify nuclei.
Murine tonsil tissue was used as a positive control for the staining of capillaries and
arterioles.

Arterioles and capillaries were counted on transverse sections from each hindlimb in
a blinded manner in 5 randomly selected high-power fields at 10X magnification. Vascular
images were captured by using an inverted light microscope (Olympus IX71) and were
analyzed by using Image-Pro Plus software (Media Cybernetics, Rockville, MD). Vessel
density was expressed as the number of arterioles or capillaries per mm<sup>2</sup>. Anti–Ki-67
staining (dilution 1:50; DAKO IgG1) was used to determine the degree of cell proliferation in vivo.
**Immunofluorescence Studies and Determination of Cell Engraftment Rate**

At 21 days after the injection of PBS or GFP+ AT-MSCs (mock-transduced or transduced with TERT and MYOCD) into the ischemic hindlimbs of ApoE−/− mice, the hindlimbs were harvested for analysis, and the number of GFP+ cells was quantified. Hindlimb cryosections (5 µm, 20 sections/leg) from each animal were incubated with anti–smooth muscle α-actin antibody (Sigma Aldrich) overnight at 4°C. Sections were then incubated with phycoerythrin (PE)-conjugated anti-rabbit IgG (Invitrogen) to stain for actin. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Immunostained tissues were visualized by using a Cambridge Research & Instrumentation (CRI) Nuance multispectral imaging system (Woburn, MA; http://www.cri-inc.com). A spectral cube for cells was collected containing the complete spectral information at 10-nm wavelength intervals from 520 to 720 nm. The resulting images were unmixed by using the Nuance system to obtain 3 images that each correspond to the fluorochrome GFP, PE (actin), or DAPI. The number and distribution of mock-transduced AT-MSCs or AT-MSCs transduced with TERT and MYOCD were determined by counting cells positive for GFP and DAPI staining. The number and location of smooth muscle α-actin–positive cells were determined by counting cells positive for PE.
Supplemental Figures

Supplemental Figure I. Lentiviral expression of TERT and MYOCD in adipose tissue–derived mesenchymal stromal cells (AT-MSCs). A, Schematic representation of the transducing vector construct. B, Yellow fluorescent protein (YFP) expression in transduced AT-MSCs, as measured by flow cytometry. Multiplicity of infection (MOI) ranged from 1 to 20. C, MYOCD expression in mock- MYOCD-transduced AT-MSCs, as determined by means of Western blot analysis using anti-myocardin antibody. D, MYOCD-V5 epitope fusion protein expression in mock- and MYOCD-V5 epitope–transduced AT-MSCs, as determined by means of Western blot analysis using anti-V5 epitope antibody. E, MYOCD expression in heart (positive control) and liver (negative control) tissue from C57/BL6 mice. P<sub>CMV</sub>, cytomegalovirus promoter; MYOCD, myocardin; TERT, telomerase reverse transcriptase; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; V5,
gene construct encoding the V5-epitope; YFP, gene construct encoding the yellow fluorescence protein; \( P_{SV40} \), promoter SV40; \( \Delta U3 \), deleted region of the 3'-LTR, which allows for biosafety of the vector; 3'-'-LTR, 3'-Long Terminal Repeats for viral packaging; Blast, blasticidin resistance gene; Amp, ampicillin resistance gene; \( P_{RSV} \), Rous Sarcoma Virus (RSV) enhancer/promoter; \( /5'-'-LTR \), 3'-Long Terminal Repeats for viral packaging; \( \psi \), psi packaging sequence for viral packaging; RRE, HIV-1 Rev (Human immunodeficiency virus type 1 Regulator of Virion) response element; cPPT, central polypurine tract.
Supplemental Figure II. Clonogenic assessment of adipose tissue–derived mesenchymal stromal cells (AT-MSCs) with and without lentiviral expression of TERT and MYOCD. Colonies derived from single AT-MSCs harvested from aged (12-month-old) and young (1-month-old) C57 or ApoE<sup>−/−</sup> mice. AT-MSCs were nontransduced (Nt), mock-transduced (Mock), or transduced with MYOCD, TERT, or MYOCD+TERT. Panels show representative phase-contrast photomicrographs of colonies in methylcellulose at day 15 (magnification=5x). Images are representative of 3 independent experiments with 5 mice in each group. ApoE<sup>−/−</sup>, apolipoprotein E–deficient mice; MYOCD, transduced with pLenti-myocardin vector; TERT, transduced with pLenti-telomerase reverse transcriptase vector.
Supplemental Figure III. Myogenesis in adipose tissue–derived mesenchymal stromal cells and bone marrow mesenchymal stromal cells from aged C57/BL6 mice transduced with or without TERT and/or MYOCD. A, Western blot analysis of cardiac actin and smooth muscle α-actin expression in adipose tissue–derived mesenchymal stromal cells (AT-MSCs) and bone marrow MSCs (BM-MSCs) from aged (12-month-old) C57/BL6 (C57) mice that were nontransduced (Nt), mock-transduced, or transduced with TERT and/or MYOCD (n=5 mice per group). As a control, analysis of smooth muscle α-actin expression was performed in human bone marrow mesenchymal stem cells (designated here as hMSCs) that were nontransduced, mock-transduced, or transduced with TERT and/or MYOCD. As a loading control, proteins were stripped and re-incubated with GAPDH antibody. B, Densitometric analysis of the protein bands shown in (A). Results are representative of 3 different experiments, and data are presented as the mean ± standard
deviation. *$P<0.05$ and **$P<0.01$ vs. mock-transduced AT-MSCs, BM-MSCs, or human mesenchymal stem cells.
Supplemental Figure IV. Structural analysis of 12-month-old ApoE\(^{-}\) mice after hindlimb ischemia and cell transplantation. Representative hematoxylin and eosin-stained cross-sections of muscle from the (A) ischemic (ligation, right limb) and (B) non-ischemic (no ligation, left limb) limbs after injection with phosphate-buffered saline (PBS) or with mock- or TERT and MYOCD–transduced GFP\(^{+}\) AT-MSCs (magnification=10x). Scale bar=100 \(\mu m\).
Supplemental Figure V. Fluorescence microscopy analysis of skeletal muscle from ApoE\(^{-/-}\) mice 21 days after the transplantation of lentivirally transduced adipose tissue–derived mesencymal stem cells (AT-MSCs) from 12-month-old green fluorescent protein (GFP)-expressing mice. Representative fluorescence microscopy images of tissue sections showing the retention of (A) mock-transduced GFP\(^{+}\) AT-MSCs and (B) TERT and MYOCd–transduced GFP\(^{+}\) AT-MSCs at the injection site 21 days after transplantation (magnification=5x and 10x).
Supplemental Figure VI. Evaluation of proliferation by using the Ki-67 marker after hindlimb ischemia and cell transplantation. **A**, Representative Ki-67–stained cross sections of muscle from ischemic (ligation, right limb) and non-ischemic (no ligation, left limb) limbs after injection with phosphate-buffered saline (PBS) or with mock- or TERT and MYOCD–transduced GFP⁺ AT-MSCs (magnification=10x). Scale bar=100 μm. **B**, Quantitative data showing the percentage of brown-stained Ki-67–positive cells (brown arrows in A) in relation to the total number of nuclei (brown stained plus blue stained, shown by blue arrows in A). Graph represents data combined from 3 independent experiments; results are presented as the mean ± standard deviation.
Supplemental Figure VII. Engraftment and vascular differentiation of TERT and MYOCD–transduced green fluorescent protein expressing (GFP⁺) adipose tissue–derived mesenchymal stromal cells (AT-MSCs) into ischemic tissue. Representative micrographs obtained with the CRi Nuance multispectral imaging system showing GFP⁺ cells co-localized with 4’,6-diamidino-2-phenylindole (DAPI) in the ischemic legs of ApoE⁻/⁻ mice at 21 days after treatment with (A) mock-transduced GFP⁺ AT-MSCs or (B) TERT and MYOCD–transduced GFP⁺ AT-MSCs. Phycoerythrin (PE)-conjugated anti-smooth muscle α-actin antibody (SM α-actin) was used to identify smooth muscle cells in cross-sections of muscle tissue. GFP⁺ cells co-localized with SM α-actin, indicating vasculature formation from transplanted cells. Results are representative of those obtained for the 5 ApoE⁻/⁻ mice in each treatment group. Magnification=10x and 20x. Scale bar=50 µm.
Online Supplemental Table. Quantitative Analysis of Arteriole and Capillary Density in the Limbs of ApoE<sup>−/−</sup> Mice After Treatment

Values represent the mean ± standard deviation vessel density (in vessels/mm<sup>2</sup>; n=5 per group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arterioles</th>
<th>Capillaries</th>
</tr>
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<tbody>
<tr>
<td>No ligation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>20±2</td>
<td>210±24</td>
</tr>
<tr>
<td>Ligation</td>
<td></td>
<td></td>
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<tr>
<td>1 day post-PBS</td>
<td>7.0±3&lt;sup&gt;†&lt;/sup&gt;</td>
<td>65.0±30&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>21 days post-PBS</td>
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<td>120±38&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>21 days post–GFP&lt;sup&gt;+&lt;/sup&gt; mock-transduced AT-MSCs</td>
<td>13±5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>165±49&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>21 days post–GFP&lt;sup&gt;+&lt;/sup&gt; TERT+MYOCD-transduced AT-MSCs</td>
<td>17±4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>204±32&lt;sup&gt;†&lt;/sup&gt;</td>
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</table>

<sup>†</sup>P<0.01 vs. the contralateral nonischemic limb (no ligation); <sup>‡</sup>P<0.05 vs. 1 day post-PBS. AT-MSCs, adipose tissue–derived mesenchymal stromal cells; TERT+MYOCD, GFP<sup>+</sup> AT-MSCs co-transduced with pLenti-telomerase reverse transcriptase (TERT) and pLenti-myocardin (MYOCD) vector; GFP, green fluorescent protein.
Supplemental References


