mTORC2 Activation by Reconstituted High-Density Lipoprotein Prevents Senescence in Circulating Angiogenic Cells

Xianrong Guo, Miao Yu, Xiaomin Kang, Hongchao Yin

Objective—Circulating angiogenic cells (CACs) participate in neovascularization and arterial repair. Although high-density lipoprotein (HDL) is known to enhance the functional activity of CACs, the mechanisms underlying this regulation are poorly understood. Here, we examined the mechanism(s) by which reconstituted HDL (rHDL) affects CAC senescence.

Methods and Results—CACs isolated from human peripheral blood and treated with rHDL displayed reduced senescence, as measured by acidic β-galactosidase staining. This protective effect was blocked by the mammalian target of rapamycin (mTOR) inhibitor (rapamycin). According to Western blot analysis and immunoprecipitation results, rHDL promoted mTOR phosphorylation, mTOR-rictor complex formation, and mTOR-rictor–dependent Akt activation, which were accompanied by increased nuclear translocation of human telomerase reverse transcriptase and enhanced nuclear telomerase activity. Suppression of rictor gene expression with a small interfering RNA blocked mTOR-rictor complex formation and Akt activation. The suppression also abolished the rHDL-induced inhibition of CAC senescence and promotion of nuclear telomerase activity. Treatment of aged mice with rHDL attenuated spleen-derived CAC senescence. In CACs isolated from rHDL-treated aged mice, the phosphorylated mTOR and Akt levels were significantly enhanced.

Conclusion—rHDL stimulates sustained mTOR phosphorylation and mTOR-rictor complex formation and inhibits senescence onset in CACs through mTOR complex-2 pathway activation. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis • cardiovascular disease prevention • endothelial function • signal transduction • vascular biology
mTORC1 and mTORC2, have been identified, both of which contain phosphorylated mTOR as their central component. The mTORC1 complex composed of mTOR and raptor is highly sensitive to rapamycin, and phosphorylates p70-S6 kinase 1 to activate protein synthesis. Activation of mTORC1 by various stimulating factors, including insulin, primarily occurs through the phosphatidylinositol 3-kinase/Akt pathway. Therefore, mTORC1 plays a key role in insulin signaling and in maintaining metabolic homeostasis. Activated mTORC1 phosphorylates downstream targets (including p70 S6K on Thr389 and 4E-BP1 on Thr229) that regulate translational initiation, ribosome biogenesis, and other growth or proliferation events.15,16

The mTORC2 complex composed of mTOR, rictor, G-protein β-subunit like protein, and mammalian stress-activated protein kinase interacting protein 1 is regulated by growth factors, nutrient levels, and other factors. mTORC2 exerts effects on its downstream targets, including Akt, a protein kinase that mediates the telomerase activity and function of prosurvival genes and cell-cycle regulators.17,18 Indeed, mTORC2 exerts most of its cell-survival effects through its direct phosphorylation of Ser473 in the hydrophobic motif of Akt.19,20 Although studies with acute exposure to rapamycin led researchers to initially conclude that mTORC2 is rapamycin insensitive, later studies have shown that long-term rapamycin exposure inhibits mTORC2 formation and blocks signal transduction through activated mTOR.23

To date, the functional significance of mTOR signaling has not been well characterized in CACs. Therefore, the purpose of this study was to explore whether reconstituted HDL (rHDL) influences CAC senescence through the mTOR signaling pathway.

Materials and Methods
This section provides an abbreviated description of the Materials and Methods used in this study. A detailed description of all of the methods is provided in the Supplemental Materials and Methods, available online at http://atvb.ahajournals.org.

Reagents
rHDL was isolated and prepared from sera collected from young, healthy individuals, as described previously.24 Monoclonal antibodies against phospho-Akt were from Santa Cruz Biotechnology. Unless otherwise noted, all other chemicals were from Sigma-Aldrich.

Cell Culture
CACs were prepared and cultured as previously described.8,25,26 Briefly, total mononuclear cells were isolated by density gradient centrifugation from the peripheral blood of healthy volunteers. Immediately after isolation, mononuclear cells (4×10⁶) were plated onto human fibronectin–coated culture dishes. The cells were main-
performed as previously described. Cells were seeded with or without CACs (siRNAs) were transfected into CACs as described. For a further kit (Roche). Rictor, raptor, and control small interfering RNAs the Telo TAGGG Telomerase polymerase chain reaction ELISA plus Total RNA was extracted and purified using an RNeasy RNA kit (QIAGEN). Western blotting and immunoprecipitation were performed as previously described.

**Acidic β-Galactosidase Staining**

Senescence-associated acidic β-galactosidase (β-gal) staining was performed as previously described. Cells were seeded with or without rHDL (10, 50, 100, or 150 μg/mL) and were incubated with rapamycin or Akt IV inhibitor for 7 days. Cells were fixed for 10 minutes in 2% formaldehyde and 0.2% glutaraldehyde in PBS and were incubated at 37°C for 12 hours with fresh β-gal solution.

**Measurement of Mitochondrial Membrane Potential**

To confirm the onset of cellular senescence using a second method, the electrochemical potential (ΔΨ) of the CAC mitochondrial membrane was measured using confocal microscopy with the lipophilic cationic probe fluorochrome JC-1. In living cells, JC-1 accumulates in the mitochondria in a potential-dependent manner. At high membrane potentials, JC-1 forms J-aggregates (emission at 590 nm) and produces red fluorescence. At low membrane potentials, JC-1 continues to exist as a monomer (emission at 530 nm) and produces green fluorescence.

**Western Blotting Analysis and Immunoprecipitation**

Western blots and immunoprecipitation were performed as previously described.

**RNA Isolation, Telomerase Activity Assay, and Small Interfering RNA Transfection Techniques**

Total RNA was extracted and purified using an RNeasy RNA extraction kit (Qiagen). Telomerase activity was measured using the Telo TAGGG Telomerase polymerase chain reaction ELISA kit (Roche). Rictor, raptor, and control small interfering RNAs (siRNAs) were transfected into CACs as described. For a further description of these methods, please see the Supplemental Materials and Methods.

**Results**

Rapamycin Abolishes the rHDL-Induced Inhibition of CAC Senescence

Acidic β-gal serves as a biochemical marker for cellular senescence. Consistent with a previous study, the CACs started to become senescent after 7 days of cultivation (Figure 1A and 1B). This finding was confirmed by the ΔΨ results (Figure 1C and 1D). Treatment with 50, 100, or 150 μg/mL rHDL...
rHDL reduced the number of acidic β-gal<sup>+</sup> CACs (Figure 1E) and improved the ΔΨ of CACs (data not shown). Coincubation with 1 or 5 nM rapamycin abrogated the rHDL-mediated reduction in acidic β-gal<sup>+</sup> CAC levels (Figure 1F), suggesting that rHDL inhibits CAC senescence via the mTOR signaling pathway.

**rHDL Promotes mTOR-Ser2448 Phosphorylation and mTOR Nuclear Translocation in CACs**

The mTOR phosphorylation at Ser2448 and Ser2481 was observed in the presence or absence of rapamycin. Treatment with rHDL markedly increased mTOR phosphorylation at Ser2448 but not at Ser2481 (Figure 2A). Ser2448 phosphorylation peaked at 4 hours and remained at a high level at 24 hours. Rapamycin reduced the active effect of rHDL on Ser2448 phosphorylation, but the total mTOR protein levels were not affected by rHDL (Figure 2B).

An additional regulatory mechanism of mTOR signaling may occur via cytoplasmic-nuclear shuttling. Therefore, we also examined whether rHDL influenced the nuclear localization of mTOR phosphorylated Ser2448. After CACs were cultured with rHDL for 4 hours, mTOR phosphorylated Ser2448 was observed in the nucleus fraction using Western blot. This nuclear localization was inhibited by rapamycin (Figure 2C and 2D). Immunohistochemistry showed a similar result (see Supplemental Results).

**rHDL Induces mTORC2-Dependent Akt-Ser473 Phosphorylation**

The phosphorylation of Akt at its primary phosphorylation site (Ser473) was analyzed during CAC incubation with rHDL for up to 24 hours. Akt-Ser473 phosphorylation increased after 10 minutes and peaked after ~4 hours (Figure 3A and 3B), whereas the total Akt level remained unchanged. Rapamycin inhibited Akt phosphorylation starting at 4 hours, with inhibition increasing with longer incubation times (Figure 3B). When CACs were coinoculated with HDL and an Akt IV inhibitor, the Akt inhibition abrogated the rHDL inhibitory effect (Figure 3C). These results indicate that the rHDL-mediated inhibition of CAC senescence is mTOR/Akt dependent.

The roles of mTORC1 and mTORC2 in the inhibitory effect of rHDL on CAC senescence were analyzed. The mTORC2 complex is known to phosphorylate Akt at Ser473. To confirm whether rHDL induces mTORC2 formation in CACs, coexpression and association between mTOR and rictor were assessed in CACs treated with rHDL alone or cotreated with rapamycin. mTOR formed a complex with rictor in rHDL-treated CACs, whereas rapamycin inhibited the coprecipitation of both proteins (Figure 4A). Western blot analysis revealed that the rictor and raptor protein levels were consistently reduced (up to 85%) by CAC transfection with rictor- or raptor-specific siRNAs, respectively. Control siRNA had no effect (Figure 4B). The siRNA-treated CACs next were cocultured with rHDL for 4 hours. The siRNA-mediated suppression of rictor gene expression significantly decreased the rHDL-induced Akt phosphorylation compared with control siRNA transfection, whereas siRNA-mediated raptor suppression had no effect (Figure 4C). Suppression of rictor gene expression also abolished the inhibitory effect of rHDL on CAC senescence (Figure 4D and 4E).

Taken together, these findings suggest that rHDL stimulates mTOR-rictor complex formation and downstream Akt activation in CACs. Prolonged exposure of CACs to rapamycin inhibits Akt phosphorylation by inhibiting mTORC2, and rHDL prevents CAC senescence through the mTORC2/Akt signaling pathway.

**rHDL Enhances the Nuclear Translocation of hTERT and Nuclear Telomerase Activity in CACs Through the mTORC2/Akt Pathway**

Cellular senescence is essentially influenced by telomerase activity, which is regulated at the hTERT expression, phosphorylation, and nuclear translocation levels. To explore the
mTORC2/Akt downstream mechanisms, we examined whether rHDL affected hTERT mRNA and protein expression in CACs using semiquantitative reverse transcription-polymerase chain reaction and Western blot (Figure 5A). Averaged densitometric quantification showed that the hTERT mRNA and protein levels in CACs were not changed significantly by rHDL (data not shown). We therefore explored whether rHDL-induced mTORC2 and Akt activation influenced hTERT nuclear translocation in CACs. Nuclear accumulation of hTERT (Figure 5B) and nuclear telomerase activity (Figure 5E) in CACs were promoted by rHDL. These effects were blocked by rapamycin, by Akt IV inhibitor, and by siRNA-mediated suppression of rictor gene expression (Figure 6C to 6E).

**Treatment With rHDL Reduces CAC Senescence and Increases CAC Number in the Spleen of Aged Mice**

To study whether rHDL can affect CAC senescence in vivo, we measured the senescence of CACs isolated from the spleen of aged (16 to 18 months) and young (2 to 3 months) mice. In aged mice, the percentage of splenic senescent cells was ≈3-fold higher and the number of splenic CACs was reduced by ≈0.4-fold compared with young mice (Figure 6A). Treatment of aged mice with rHDL for 6 days reduced the CAC senescence to 11.5% (control, 22.8% for 4.9%) and increased the number of CACs to 76±10.8 cells/high-power field compared with mice treated with PBS (53±6.7 cells/high-power field) (Figure 6B and 6C).

To further explore the effect of rHDL on CACs in vivo, phosphorylated mTOR and Akt levels were detected and the nuclear telomerase activity was measured. Treatment with rHDL significantly enhanced the phosphorylation levels of mTOR and Akt and the nuclear telomerase activity in CACs isolated from aged mice (Figure 6D and 6E). The migratory capacity (Figure 6F) and adhesion activity (Figure 6G) of CACs isolated from rHDL-treated aged mice (67.0±5.1 and 43.8±7.3 cells/high-power field, respectively) were improved significantly compared with mice treated with PBS (43.2±3.8 and 27.6±4.4 cells/high-power field, respectively).
Discussion

We showed here that rHDL inhibits the onset of CAC senescence by promoting sustained mTOR phosphorylation, mTORC-rictor complex formation, and mTORC2-dependent Akt phosphorylation. Furthermore, mTORC2-mediated Akt activation enhances the nuclear translocation of hTERT and nuclear telomerase activity in CACs. These findings reveal important mechanistic clues in understanding the effect of HDL on CACs.

The antiatherosclerotic actions of HDL are generally attributed to its uptake of cellular cholesterol from the periphery and its transport of excess cholesterol to the liver. Recently, HDL was shown to have multiple direct effects on EPCs that may be critical for atheroprotection. For example, HDL increases the number of EPCs, perhaps by prolonging their survival period or preventing senescence. Other effects include enhancing progenitor-mediated endothelium repair in mice, promoting the differentiation of mononuclear cells/EPCs into endothelial-like cells, and enhancing ischemia-induced angiogenesis. Low plasma HDL levels clinically are associated with impaired endothelial function and a decreased number of EPCs. Increasing the HDL level in patients with type 2 diabetes by systemic rHDL infusion can improve the availability of CD34+ vascular endothelial growth factor receptor 2+ cells (which may contain CACs).

It is widely accepted that CACs and EPCs collaboratively participate in angiogenesis and arterial repair. Furthermore, the improvement of CAC function is very important for neovascularization and antiatherogenesis. However, little is known about the molecular mechanisms by which HDL regulates CAC function. We observed that rHDL potently delayed senescence onset in CACs through a mechanism involving mTOR activation. mTOR activity is regulated by phosphorylation at Ser2448 or autophosphorylation at Ser2481. In this study, rHDL appeared to activate mTOR signaling by promoting a rapid and sustained high-level phosphorylation of mTOR Ser2448 but not Ser2481. In terms of whether rHDL activates Akt through activated mTOR, we observed that rHDL increased and maintained a high level of Akt-Ser473 phosphorylation. Incubation of CACs with rapamycin for 4 hours inhibited mTOR-Ser2448 phosphorylation and completely attenuated Akt-Ser473 phosphorylation. Inhibiting Akt with a special inhibitor also abrogated the rHDL inhibitory effect on CAC senescence.

mTORC1 formation is increased in vascular endothelial cells mainly during the early stage of cell stimulation (e.g., hypoxic state for <3 hours), whereas mTORC2 formation is delayed. We observed that mTORC2 formation was increased but mTORC1 was nearly undetectable after CACs were incubated with rHDL for 240 minutes. Rapamycin blocked mTOR-rictor complex formation and simultaneously abolished rHDL-induced nuclear translocation of hTERT. Blots are representative of 3 independent experiments.

Figure 5. rHDL enhanced hTERT nuclear translocation and nuclear telomerase activity. A, Reverse transcription–polymerase chain reaction (RT-PCR) and Western blot of CACs treated with rHDL (50 μg/ml) for 24 hours. Averaged densitometric quantification revealed that rHDL did not alter the hTERT mRNA or protein level (data not shown). B to D, Nuclear and cytosolic fractions of CACs prepared as in A were subjected to immunoblotting with an anti-hTERT antibody. Histone and GAPDH served as loading controls. Coincubation of rHDL with rapamycin (rapa; 1 nmol/L) or Akt IV inhibitor (Akt; 1 μmol/L) (C) or treatment with a rictor-specific siRNA (D) abolished rHDL-induced nuclear translocation of hTERT. Blots are representative of 3 independent experiments. E, Telomerase activity was measured in the nuclear fraction of CACs treated as above. The rHDL-induced increase of nuclear telomerase activity in CACs was reversed by suppression of rictor gene expression or treatment with rapamycin (1 nmol/L) or Akt IV inhibitor (1 μmol/L). Mean ± SEM, *P < 0.05, n = 6.
Akt pathway. Determination of whether rHDL can enhance mTORC1 formation in CACs at the early stage (<4 hours) of incubation requires further investigation.

The proatherosclerosis factors oxidized low-density lipoprotein, angiotensin II, and homocysteine reportedly accelerate CAC senescence onset by inactivating telomerase activity.9,41,42 Conversely, estrogen and resveratrol reduce CACs senescence by augmenting telomerase activity.43,44 The overexpression of hTERT by adenovirus-mediated gene delivery also delays CACs senescence.45 We observed that rHDL had no effect on the hTERT mRNA or protein level in CACs, suggesting that rHDL did not regulate telomerase activity at the hTERT-expression level. Because telomerase activity also can be positively regulated by Akt-mediated phosphorylation and hTERT nuclear translocation.46,47 we explored whether rHDL-induced mTORC2/Akt activation influences hTERT nuclear translocation. rHDL promoted hTERT accumulation in the nucleus, significantly increasing the nuclear telomerase activity of CACs. This effect was blocked by rapamycin and the Akt IV inhibitor, as well as by the suppression of rictor gene expression. These results suggest that rHDL increases the nuclear telomerase activity in CACs through mTORC2 and Akt activation. Because Akt also regulates endothelial nitric oxide synthase activity and some cell cycle regulators and cell survival pathways, other mechanisms may contribute to the inhibitory effect of rHDL on CAC senescence.

To study whether rHDL has an effect on CACs in vivo, we measured the senescence of CACs isolated from the spleens of aged and young mice. Compared with the young mice, the aged mice displayed a higher percentage of senescent cells and significantly fewer isolated CACs. Treatment of aged mice with rHDL for 3 days did not affect CAC senescence, although rHDL treatment for 6 days attenuated CAC senescence and significantly increased the number of CACs compared with PBS-treated mice. In CACs isolated from rHDL-treated aged mice, the phosphorylated mTOR and Akt levels and telomerase activity were significantly enhanced. Consistently, functional activity assays showed that the migratory capacity and adhesion activity of CACs isolated from rHDL-treated aged mice were significantly improved. These results indicate that rHDL administration inhibits CAC senescence and improves CAC activity in vivo.
In conclusion, rHDL was shown to stimulate mTOR phosphorylation, mTORC2 formation, and downstream Akt activation in CACs and to inhibit CACs senescence onset through mTORC2/Akt–mediated enhancement of nuclear telomerase activity. These results implicate novel mechanisms by which rHDL protects the function of CACs, and they indicate mTORC2 as an important mediator of CAC viability.

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Disclosures

None.

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Supplement Material

Reagents

Reconstituted HDL was isolated and prepared from sera collected from young healthy individuals, as described previously\(^1\). The mAbs against phospho-Akt were from Santa Cruz Biotechnology. Unless otherwise noted, all other chemicals were from Sigma.

Cell Culture

Circulating angiogenic cells were prepared and cultured as previously described\(^2,3\). Total mononuclear cells were isolated by density gradient centrifugation from the peripheral blood of healthy volunteers. Immediately after isolation, mononuclear cells (4 × 10\(^6\)) were plated onto human fibronectin–coated culture dishes and were maintained in EBM-2 (Clontech) supplemented with VEGF, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and 5% fetal calf serum (FCS). After 4 d in culture, nonadherent cells were removed by washing with PBS and new media was applied. Adherent CD31\(^+\)CD45\(^+\) cells positive for VEGFR2 (VEGFR2\(^+\)), AcLDL uptake, and Ulex europaeus agglutinin I (UEA-1) binding were designated as CACs. The mTOR inhibitor (rapamycin) and Akt IV inhibitor (Alexis Corp., San Diego, California) were used at subcytotoxic concentrations, according to a cell viability assay (Cell Titer-Blue\(^\text{TM}\), Promega).

Acidic β-gal Staining

Senescence-associated acidic β-gal staining was performed as previously described\(^4\). Mononuclear cells were incubated in EBM supplemented with 5% FCS. On day 4, adherent cells were seeded with or without rHDL (10, 50, 100, or 150 μg/ml) and were coincubated with
rapamycin or Akt IV inhibitor for 7 d. Cells were fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS and were incubated at 37 °C for 12 h with fresh β-gal solution. Cells were counterstained with DAPI for 10 min. The absolute number of β-gal+ cells was determined based on 1000 counted cells.

**Measurement of Mitochondrial Membrane Potential**

The lipophilic cationic probe fluorochrome JC-1 (Molecular Probes, Carlsbad, CA) was used to explore changes in the mitochondrial membrane potential (Δψ) of cultured CACs. The amount of JC-1 to enter the mitochondria varies in proportion to the membrane potential. JC-1 forms J-aggregates at the higher intramitochondrial concentrations induced by higher mitochondrial membrane potential values. JC-1 exists as either a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. Cells were cultured for 1, 3, 5, or 7 d, rinsed with medium, and loaded with JC-1 (5 µM). After 20 min of incubation at 37 °C, cells were examined by confocal microscopy (Bio-Rad) using an argon laser to excite JC-1 at 488 nm. Emissions were recorded simultaneously at 530 nm for JC-1 monomer and 590 nm for J-aggregates into independent detectors. Ten cells were selected within a single image to analyze the fluorescence intensity ratios using Image-Pro 5.1 software (MediaCybernetics, MD).

**Western Blotting Analysis and Immunoprecipitation**

The CACs were lysed with extraction buffer (pH 7.5, 20 mM HEPES, 1% Nonidet P-40, 10% glycerol, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, and complete proteinase inhibitor cocktail [Roche]). Cellular proteins (50 µg/lane) were
fractionated using 10% SDS-polyacrylamide gel electrophoresis. Western blotting was performed using mAbs against Akt (Santa Cruz), mTOR, rictor, raptor (Cell Signaling), and polyclonal antibodies against hTERT (Santa Cruz). Chemiluminescence was performed, and protein bands were analyzed by densitometric quantification using ImageJ 1.31v software.

Immunoprecipitation was performed as described\(^8\). Briefly, CACs were incubated with rHDL in the presence or absence of rapamycin for 4 h, rinsed in cold PBS, and lysed in ice-cold lysis buffer (0.3% CHAPS, 40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 µg/ml leupeptine, 10 µg/ml aprotinin, 1 mM sodium orthovanadate). The lysates were centrifuged at 4 °C for 15 min and the supernatants were collected. Immunoprecipitation was performed with 500 µg of total protein incubated with 3 µg of anti-mTOR antibody for 90 min at 4°C. Immunocomplexes were captured with protein A-Sepharose beads (Amersham Biosciences), washed 4 times in CHAPS-containing buffer, resuspended in a protein loading buffer containing SDS (Boston Bioproducts), and boiled for 5 min. Immunoprecipitated proteins were separated using a polyacrylamide gel and analyzed by Western blot.

To detect hTERT nuclear translocation, the nuclear and cytosolic CAC fractions were separated using a commercially available kit (Pierce). Western blotting was performed using an anti-hTERT polyclonal antibody.

**Immunohistochemistry**

The CACs were incubated with or without rapamycin on coverslips in an HDL-containing medium for 4 h. To identify nuclear translocation of mTOR, CACs were fixed, permeabilized, blocked with 3% BSA, and incubated with an anti-human phospho-mTOR antibody (Cell Signaling) overnight at 4 °C. After washing, cells were incubated with a rhodamine-conjugated
secondary antibody at room temperature for 30 min, counterstained with DAPI, and observed by
confocal microscopy.

**RNA isolation and RT-PCR**

Total RNA was extracted and purified using an RNeasy RNA extraction kit (Qiagen). Equal
amounts of RNA were reverse-transcribed into cDNA with the iScript cDNA synthesis kit
(Bio-Rad). Primers for hTERT (sense: 5’-GCCTAAGCTG TACTTTATCA A-3’; antisense:
5’-CGCAAACAGC TTGTTCTCCA TGTC-3’) were used to generate the 457-bp PCR product.
A GAPDH fragment was amplified as an internal control.

The PCR amplification included an initial denaturation at 94 °C for 5 min, 28 cycles of
denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and extension for 30 s, and a final
extension of 5 min at 72 °C. The PCR products were analyzed using an ethidium
bromide-stained 1% agarose gel. Gel bands were semiquantified using Quantity One 1D
Analysis Software Version 4.4 (Bio-Rad). Representative results of 4 independent RT-PCR
experiments are shown.

**Telomerase Assay**

Harvested cells were seeded into a 6-well cultured plate and incubated with rHDL, with or
without rapamycin and/or Akt IV inhibitor, for 24 h. After washing with PBS, nuclear fractions
of CACs were separated as described above. Supernatant protein concentrations were determined
using a Bradford assay. Telomerase activity was measured with 3 µg of protein using the Telo
TAGGG Telomerase PCR ELISAplus kit (Roche).
Transfection of siRNA into CACs

Rictor, raptor, and control siRNAs were transfected into CACs as described\(^9\). Briefly, CACs (2 × 10^5/well in 6-well plates) were incubated in antibiotic-free ECM medium (2 mL) to 60% confluency. Cells were incubated for 7 h with siRNA transfection medium (1 mL/well) containing siRNA (80 pmol/well) and siRNA transfection reagent (8 µL/well). Transfected CACs were cultured in ECM medium for an additional 24 h. Rictor and raptor expression levels were analyzed by Western blotting.

Isolation of CACs from Mouse Spleen

Spleens from mice were explanted and mechanically minced, and mononuclear cells were isolated using a Ficoll gradient (Lympholite-M, Cedarlane) as described previously\(^{10,11}\). The mononuclear cells were cultured on fibronectin as described above. After 4 d in culture, cells were washed with PBS and the adherent CACs were identified by uptake of Di-LDL and UEAI binding. The numbers of DiLDL- and UEAI-positive cells were evaluated by two independent investigators using fluorescence microscopy.

Cellular Adhesion Assay

Adhesion activity was evaluated as described previously\(^12\). Briefly, EPCs (2.5 × 10^4 cells/well) in 5% FBS/EBM-2 medium were seeded onto 96-well plates precoated with vitronectin, collagen type I, fibronectin, and laminin. The cells were incubated for 1 h at 37 °C and then washed 3 times with PBS. The attached cells were fixed and stained with DAPI and visualized under a fluorescent microscope (10× magnification). The numbers of adhered cells were counted in 8 wells, and adhesion activity was reported as the mean number of attached cells.
Cellular Migration Assay

The migratory capacity of freshly isolated CACs was investigated using the modified Boyden chamber assay as described previously\textsuperscript{12}. In brief, cells suspended in EBM-2 medium were placed in the upper chamber (5 × 10\textsuperscript{4} cells/chamber), and the lower chamber was filled with medium containing 50 ng/mL recombinant mouse VEGF protein (R&D Systems, Inc., Minneapolis, MN, USA). The chamber was incubated for 24 h at 37 °C. The EPCs that had migrated into the lower chamber were fixed with 2% PFA/PBS for 10 min and stained with DAPI. Migrated cells were viewed under a fluorescent microscope (40× magnification) and counted in 4 chambers, with 5 HPFs per chamber. Migration activity was reported as the mean number of migrated cells.

![Control, rHDL, rHDL+rapa](image)

**Supplemental Figure I.** Immunohistochemistry revealed that mTOR–P-Ser2448 (red stain) levels in the nucleus (DAPI stain) were increased after CACs were cultured with rHDL (50 µg/mL) for 4 h. No signal was detected in CACs coincubated with rapamycin (rapa, 1 nM).

**Supplemental References**

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