Mthfr deficiency induces endothelial progenitor cell senescence via uncoupling of eNOS and downregulation of SIRT1

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Hyperhomocysteinemia (HHcy) has been recognized as a major risk factor of cardiovascular disease (20). Methylentetrahydrofolate reductase (Mthfr) plays a key role in the remethylation cycle, converting homocysteine to methionine (37). A common variant in Mthfr, C677T, is associated with decreased enzyme activity leading to HHcy in humans (10, 17). The lack of this enzyme leads to the accumulation of homocysteine and to HHcy. Although the mechanism by which HHcy injures the vessel wall and induces atherosclerosis is poorly understood, a growing body of evidence has suggested that endothelial dysfunction plays a major role (40).

Endothelial dysfunction ultimately induces an imbalance between the severity of injury and the capacity for repair (13). A variety of evidence has suggested that circulating endothelial progenitor cells (EPCs) constitute one aspect of the repair process (13, 35). Indeed, EPCs are regarded as having a key role in the maintenance of endothelial integrity and the replacement of apoptotic or damaged endothelial cells in response to various cardiovascular risk factors (2, 26). EPCs are bone marrow-derived mononuclear cells that are lineage negative and express the surface marker stem cell antigen (Scal) and the endothelial marker VEGF receptor 2 (flk-1 in mice) or CD31. Additionally, EPCs take up low-density lipoprotein and bind lectin. EPCs contribute to vascular repair, although their exact mechanism of action is still controversial. Most likely EPCs enhance the reparative capacity of the endothelium adjacent to the injury by releasing paracrine factors (14, 25, 41).

Patients with coronary artery disease exhibit reduced levels and functional impairment of EPCs (12, 13, 32, 38), severe endothelial dysfunction, and reduced bioavailability of endothelium-derived nitric oxide (NO), due to the presence of cardiovascular risk factors, advanced age, or both (4, 24, 34, 42). We previously showed (33) that mild HHcy was associated with increased generation of reactive oxygen species (ROS) in the aortic wall, which could not be reduced by treatment with antioxidants such as vitamin C. The oxidative environment has been shown to be critical for the differentiation and the mobilization of EPCs (25). Moreover, ROS are important mediators of DNA damage that leads to cellular senescence.

In the present study, we hypothesized that mild HHcy would affect EPC numbers and function by inducing premature senescence, which could account for HHcy-induced endothelial dysfunction.

METHODS

Animal experiments. All experimental procedures were approved by the Animal Care Committee of the Lady Davis Institute for Medical Research, McGill University, and followed the recommendations of the Canadian Council of Animal Care. Mice heterozygous for disruption of the Mthfr gene presenting mild HHcy were generated at the Montreal Children’s Hospital Research Institute as reported previously (7). Heterozygous Mthfr-deficient (Mthfr+/−) mice and wild-type control mice were obtained by mating Mthfr−/− mice with wild-type BALB/cAnN.CtrBR mice (Charles River). Female Mthfr+/− mice and littermate wild-type control mice aged 12–14 wk were studied. We have already shown (7, 33) that Mthfr−/− mice have a 1.5- to 2-fold elevation in plasma homocysteine (7.7 ± 0.5 mmol/l) compared with wild-type mice (4.3 ± 0.3 mmol/l).

Blood collection and mononuclear cell extraction. Blood from wild-type and Mthfr+/− mice was collected from the heart in EDTA tubes. RPMI was added to the blood (1:1 vol/vol), and mononuclear cells were isolated by centrifugation on a Ficoll gradient. Mononuclear cells then underwent flow cytometry analysis.
**Bone marrow mononuclear cell culture.** Bone marrow mononuclear cells (BM-MNCs) were obtained by flushing tibia and femur of wild-type and Mthfr<sup>-/-</sup> mice. Low-density mononuclear cells were then isolated by centrifugation on a Ficoll gradient. BM-MNCs (1.5 × 10⁶/ml) were seeded on 11-mm cell culture dishes coated with bovine plasma vitronectin (Calbiochem) and 0.1% gelatin (Sigma) and maintained in endothelial growth medium (EGM2; Cambrex). When indicated, BM-MNCs were treated with or without sepiapterin (10 μmol/l) for 7 days. Nonadherent cells were then removed, and adherent cells underwent analysis.

**Flow cytometry analysis.** Flow cytometry (Becton Coulter) was used to characterize and quantify EPCs, either extracted from blood or as cultured BM-MNCs, by the expression of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against c-kit (2B8) and phycocerythin (PE)-conjugated monoclonal antibody against Sca1 (D7) or PE-conjugated monoclonal antibody against flk-1 (BD Pharmingen).

**Immunofluorescence.** EPCs from wild-type mice were also characterized by immunofluorescence with a monoclonal CD31 antibody (Santa Cruz) and a polyclonal flk-1 (VEGF receptor-2) antibody (Abcam). As well, the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (AcLDL-Dil) was evaluated. For this evaluation, cells were incubated in medium containing AcLDL-Dil (Invitrogen, Carlsbad, CA) at 37°C for 1 h. Cells were then fixed with 2% paraformaldehyde and incubated with FITC-labeled BS-1 lectin (Sigma). Dual-positive staining for both AcLDL-Dil and BS-1 lectin characterized EPCs. EPC numbers were counted and expressed in number of cells per well. Five replicates were counted for each experimental condition. Three independent investigators evaluated the number of EPCs per well by counting three randomly selected high-power fields under epifluorescence microscopy. Results are expressed as percentages of total cell numbers.

**Western blot analysis.** For Western blot analysis, EPCs were homogenized in lysis buffer (final concentrations in PBS: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotenin, and 1 mmol/l NaVO₄). Lysates from EPCs containing 20 μg of protein were separated by electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated with anti-Sirt1 (polyclonal antibody, Upstate Biotech) overnight at 4°C. Monoclonal anti-GAPDH antibody (Novus Biologicals) was used to reprobe blots to confirm equal loading in lanes. Signals were revealed by chemiluminescence (SuperSignal West Pico chemiluminescent signal, Thermo Scientific, Rockford, IL) with the Molecular Imager Chemidoc XR5 system (Bio-Rad, Mississauga, ON, Canada) and quantified by densitometry with Quantity one software (Bio-Rad).

**Low-temperature SDS-PAGE.** EPC extracts were prepared with sodium dodecyl sulfate sample buffer in low-temperature conditions. Samples were loaded on 7.5% polyacrylamide gels and subjected to electrophoresis. Buffers and gels were cooled to 4°C, and the buffer tank was placed in an ice bath during electrophoresis. eNOS monomers and dimers were detected by Western blotting analysis using eNOS monoclonal antibody (Transduction Lab).

**Superoxide/nitric oxide measurements.** To evaluate superoxide generation, cells were incubated in the dark for 30 min at 37°C with dihydroethidium (DHE; 2 μM). The fluorescence (red signal) of oxidized DHE products was detected with a fluorescent microscope (Leica). Absolute numbers of DHE-positive cells were counted by a fluorescent microscope (Leica). Absolute numbers of DHE-positive cells were counted and expressed as DHE-positive cells per total number of cells. Intracellular superoxide levels were evaluated with 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acety ester (CM-H₂DCFDA; Invitrogen), which becomes fluorescent when oxidized to dichlorofluorescein.

Briefly, BM-MNCs were seeded on black 96-well plates with or without sepiapterin (10 μmol/l) for 7 days. After wash with PBS, cells were incubated with CM-H₂DCFDA and fluorescence was read every 2 min for 60 min with a Fluoskan FL (Thermo Fisher). Data are expressed as relative fluorescence units per 400,000 cells. NO production was evaluated with 4-amino-5-methylamino-2',7'-dichlorofluorescein diacetate (DAF-FM diacetate; Invitrogen). Absolute numbers of DAF-FM-positive cells were counted and expressed as DAF-FM-positive cells per total number of cells.

**NADPH oxidase activity.** The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity in EPC homogenates after 7 days of culture as previously described (15). NADPH (100 μmol/l) was added to the suspension (100 μl) containing lucigenin (5 μmol/l). Chemiluminescence was measured for 1 s every 6 s for 3 min in an Orion II microplate luminometer (Berthold Detection Systems, Pforzheim, Germany) and activity expressed as relative light units per minute per microgram of protein.

**Determination of cell viability by thiazolyl blue tetrazolium bromide test.** Four hundred thousand BM-MNCs were seeded in 96-well plates and incubated 7 days at 37°C and 5% CO₂ with or without sepiapterin. On the day of the experiment, cells were incubated 6 h at 37°C and 5% CO₂ with thiazolyl blue tetrazolium bromide (MTT; Sigma) at 5 mg/ml after shaking for 5 min at 150 rpm. The metabolic product of MTT (formazan) was resuspended in DMSO. Optical density was read at 560 nm.

**Quantitative RT-PCR.** Expression at the mRNA level was determined by reverse transcription (RT) followed by quantitative real-time PCR (qPCR). RNA was isolated with TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed with a Quantitect RT kit (Qiagen, ON, Canada). qPCR was performed with a Quantitect SYBR Green PCR Kit (Qiagen) with the Mx3005P real-time PCR cycler (Stratagene, La Jolla, CA). qPCR results were normalized with small ribosomal protein 16 (S16) and expressed as change relative to control. Primers were designed with

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**Fig. 1. Mthfr deficiency reduced circulating endothelial progenitor cells (EPCs) in vivo.** A: EPCs were characterized by flow cytometry analysis for the late markers of EPC differentiation, Sca1 and flk-1. The number of double-positive cells extracted from blood was significantly reduced in methylenetetrahydrofolate reductase (MTHFR)-deficient (Mthfr<sup>-/-</sup>; +/-) compared with wild-type (WT) mice (n = 4/group). CTRL, control. B: reverse transcription (RT)-quantitative real-time PCR (qPCR) showed that mRNA expression of MTHFR is significantly reduced in Mthfr<sup>-/-</sup> compared with wild-type mice (n = 5/group). S16, small ribosomal protein. Data are presented as means ± SE. *P < 0.05 vs. wild-type mice.
Primer3 (MIT Center for Genome Research, Cambridge, MA). Annealing temperature was 58°C. Primers are described in Supplemental Table S1.1.

Senescence-associated β-galactosidase activity assay. Senescent cells display increased cell size, senescence-associated expression of β-galactosidase (SA-β-gal) activity, and altered patterns of gene expression. SA-β-gal activity was measured with the β-Galactosidase Staining Kit (BioVision) according to the manufacturer’s instructions. Briefly, EPCs were washed in PBS, fixed for 10–15 min at room temperature with 0.5 ml of fixative solution, washed, and incubated overnight at 37°C with the staining solution. Cells were observed under a microscope for development of blue color. The absolute numbers of β-galactosidase-positive cells were counted and expressed as β-galactosidase-positive cells per well. SA-β-gal is present only in senescent cells and not found in presenescent, quiescent, or immortalized cells.

Telomere length. The relative telomere length was determined by qPCR as described previously (5) with minor modifications. In brief, DNA from EPCs was extracted by digestion of cells with a solution containing 25 mmol/l NaOH and 0.2 mmol/l EDTA disodium at 95°C for 1 h. The relative telomere length was then determined by qPCR, as above, with the following telomere primers: 5'-CGGTTT-GTTTGGGTTTGGTTGTTGTTGTTGTTGTTGGTT-3' and 5'-GGCTTGCCCTACCTACACCTTACACCTTACACCTTACCCCT-3'. qPCR results were normalized to β-actin gene copy determined with the primers presented in Supplemental Table S1. The results are expressed as relative telomere length per β-actin gene copy.

Supplemental Material for this article is available online at the Journal website.

**RESULTS**

*Mthfr deficiency impairs EPC formation.* The abundance of circulating EPCs was assessed in blood of wild-type and *Mthfr<sup>−/−</sup>* mice. The number of double-positive mononuclear cells for Sca1 and flk-1 was significantly decreased in *Mthfr<sup>−/−</sup>* compared with wild-type mice (Fig. 1A). To assess the functional activity of EPCs, mononuclear cells isolated from the bone marrow (BM-MNCs) have been expanded in vitro. BM-MNCs extracted from the bone marrow of wild-type mice were cultured for 7 days in a conditioned medium orienting cell differentiation into EPCs. After 7 days of culture, RT-qPCR showed that EPCs still expressed MTHFR, and this was decreased by 50% (*P < 0.05*) in the heterozygous mice compared with the wild-type cells (Fig. 1B). BM-derived EPCs expressed hematopoietic stem/progenitor cell markers Sca1 and c-kit as shown by flow cytometry (Fig. 2A) and the endothelial markers CD31 and flk-1 as shown by immunocytochemistry (Fig. 2B). The ability of *Mthfr<sup>−/−</sup>* BM-MNCs to differentiate into EPCs was reduced, as revealed by the 34% reduction (*P < 0.05*) in the number of AcLDL-Dil/BS-1 lectin double-positive cells (Fig. 2C) and the decreased number of Sca<sup>1−/−</sup>/c-kit<sup>−/−</sup> cells (Fig. 2A).

**Statistical analysis.** Data are presented as means ± SE. Within-group differences were assessed by one-way analysis of variance followed by a post hoc Student-Newman-Keuls test. A value of *P < 0.05* was considered statistically significant.

**Fig. 2.** *Mthfr* deficiency reduced EPC number. A: EPCs were characterized by flow cytometry for markers of differentiation, c-kit and Sca1. The number of double-positive cells was reduced in *Mthfr<sup>−/−</sup>* mice compared with wild-type (*n = 4/group*). B: representative images of EPCs derived from bone marrow-derived mononuclear cells (BM-MNCs) of wild-type and *Mthfr<sup>−/−</sup>* mice after 7 days of culture. EPCs were stained with CD31 and flk-1. Con-focal microscopy showed that *Mthfr* deficiency reduced the number of double-positive cells (*n = 4/group*). C: EPCs were also characterized as adherent cells with double-positive staining for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (AcLDL-Dil) and BS-1 lectin. Quantification of AcLDL-Dil and BS-1 lectin-positive cells derived from wild-type and *Mthfr<sup>−/−</sup>* BM-MNCs (right) showed that EPC number was decreased in *Mthfr<sup>−/−</sup>* mice (*n = 5/group*). Data are presented as means ± SE. *P < 0.05* vs. wild-type mice.
Mthfr deficiency induced eNOS uncoupling. DHE staining showed that ROS generation was significantly increased in EPCs from Mthfr<sup>+/−</sup> animals compared with EPCs from wild-type mice (Fig. 3A). However, there was no significant difference in NADPH oxidase activity between the two groups of mice (Fig. 3B). Measurement of intracellular ROS showed increased production in Mthfr<sup>+/−</sup> mice compared with wild-type mice. EPCs were then treated with sepiapterin, a precursor of BH<sub>4</sub>, a cofactor for endothelial nitric oxide synthase (eNOS), which significantly reduced ROS generation in cells from Mthfr<sup>+/−</sup> mice compared with untreated Mthfr<sup>+/−</sup> cells (Fig. 3C). Finally, sepiapterin improved the number of EPCs expressing the endothelial marker flk-1 in Mthfr<sup>+/−</sup> mice to a level comparable to that of wild-type animals (Fig. 3D).

The eNOS dimer-to-monomer ratio was significantly reduced in Mthfr<sup>+/−</sup> EPCs compared with those from wild-type mice, demonstrating the uncoupling of eNOS (Fig. 4A). This was associated with a significant decrease of mRNA expression of eNOS in Mthfr<sup>+/−</sup> mice compared with wild-type mice. However, mRNA levels were not affected by sepiapterin (Fig. 4B). NO production was estimated in wild-type and Mthfr<sup>+/−</sup>-derived EPCs. The DAF-FM diacetate fluorescence assay showed a significant decrease of NO production in Mthfr<sup>+/−</sup> compared with wild type. Sepiapterin treatment restored NO generation in Mthfr<sup>+/−</sup> EPCs to levels comparable to wild type (Fig. 4C). Expression levels of GTP cyclohydrolase 1 (GCH1), 6-pyrovoyl tetrabiopterin synthase (Pts), and sepiapterin reductase (Spr), genes involved in BH<sub>4</sub> biosynthesis, were determined by RT-qPCR. The mRNA expression of none of these enzymes was affected by Mthfr deficiency and/or sepiapterin treatment (data not shown).

Uncoupling of eNOS leads to EPC senescence by decreasing SIRT1 expression. A reduced EPC number could be associated with EPC senescence (3, 16). By MTT assay, viability of EPCs was equivalent in both groups of mice treated or not with sepiapterin (Fig. 5A). Acidic β-galactosidase was detected as a biochemical marker for acidification typical of cell senescence. Senescence of EPCs was increased 1.5-fold in Mthfr<sup>+/−</sup> compared with wild-type mice after 7 days of ex vivo culture. However, when cells were treated with sepiapterin, levels of EPC senescence were equivalent in wild-type and Mthfr<sup>+/−</sup> mice, as shown by β-galactosidase activity (Fig. 5B). Since

Fig. 3. Mthfr deficiency increased reactive oxygen species (ROS) generation. A: dihydroethidium (DHE) staining, representative of ROS production by EPCs, after 7 days of culture was significantly increased in Mthfr<sup>+/−</sup> compared with wild-type cells (n = 5/group). B: however, NADPH activity was comparable between wild-type and Mthfr<sup>+/−</sup> mice (n = 5/group). C: intracellular ROS generation was evaluated by 5-(and 6)-choloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA). Intracellular ROS production was increased in Mthfr<sup>+/−</sup> mice. The level of ROS was normalized by treatment of Mthfr<sup>+/−</sup> EPCs with sepiapterin, an analog of tetrahydrobiopterin (BH<sub>4</sub>), a key cofactor for endothelial nitric oxide synthase (eNOS) coupling (n = 8/group). RLU, relative fluorescence units; RLU, relative light units. D: flk-1 was used as a marker of EPC differentiation. Isotype IgG served as control (red line). The endothelial marker flk-1 was positive in every group (black line). The gated bar indicates % of flk-1 positive cells in the analysis. Quantification by flow cytometry showed that treatment of Mthfr<sup>+/−</sup> EPCs with sepiapterin improved the differentiation of cells to a level comparable to the wild type (representative of 4 different experiments). Data are presented as means ± SE. *P < 0.05 vs. wild-type mice; *P < 0.05 vs. Mthfr<sup>+/−</sup> mice without treatment.
senescence could result from a decrease in telomere length, we determined both the expression of telomerase reverse transcriptase (Tert) at the mRNA level and the relative telomere length in both groups of mice. The mRNA expression of Tert was significantly reduced in \textit{Mthfr}^{-/-} animals compared with wild-type mice, and this was prevented by sepiapterin (Fig. 5C). These results were confirmed by the significant reduction in relative telomere length of \textit{Mthfr}^{-/-} EPCs. No difference in telomere length was observed in cells from either group of mice when cells were treated with sepiapterin (Fig. 5D).

Finally, expression of SIRT1 was impaired at both mRNA and protein levels by \textit{Mthfr} deficiency. Sepiapterin prevented the decrease in mRNA and protein expression of SIRT1 (Fig. 6). These results suggest that \textit{Mthfr} deficiency by uncoupling eNOS increases ROS production, which leads to inhibition of SIRT1, then to premature senescence of EPCs, and thus eventually to endothelial dysfunction.

DISCUSSION

The present study demonstrates that \textit{Mthfr} deficiency results in increased EPC senescence, which can account for reduced number of differentiated cells after 7 days in culture. More importantly, \textit{Mthfr} deficiency induced senescence via uncoupling of eNOS, which increased the generation of intracellular ROS and decreased NO production, leading to downregulation of SIRT1 and Tert, and finally to senescence of EPCs (Fig. 7).

HHcy has been shown to be an independent risk factor for cardiovascular disease (6). Continuous damage of endothelium results in endothelial dysfunction (23) and a progressive loss of endothelial cells (18). Systemic infusion or endogenous mobilization of EPCs restores the endothelium (9, 39). As in the case of other risk factors like dyslipidemia or hypertension, we hypothesized that \textit{Mthfr} deficiency would result in a reduced number of EPCs, which could contribute to the endothelial dysfunction that we previously reported in \textit{Mthfr}^{-/-} mice (33). In agreement with previous in vitro studies, homocysteine reduced the number and differentiation of BM-MNCs into EPCs (44).

We (33) and others (27, 28, 36) have demonstrated that homocysteine in vivo increases ROS production in vascular cells, which is associated with decreased NO bioavailability. The reaction between NO and ROS generates peroxynitrite, which is elevated in the vessel wall in mild HHcy (8, 27, 28, 33).
In the present study, we found that the main source of ROS was eNOS. Thus treatment with sepiapterin, an analog of BH₄, reduced the production of ROS, which demonstrates that eNOS is the main source of superoxide anion in EPCs obtained from Mthfr+/−/− mice. Mthfr deficiency was associated with uncoupling of eNOS and subsequently with decreased NO production, which was improved by treatment with sepiapterin. The reduction of NO generation has been implicated in the induction of senescence in mature endothelial cells (22, 31). However, in vitro, NO donors such as sodium nitroprusside do not reduce the onset of EPC senescence induced during prolonged culture (45). These authors demonstrated that cell senescence is independent of NO, which is consistent with our results, as sepiapterin had no effect on the number of senescent EPCs in wild-type mice. However, it appears that exaggerated senescence observed in Mthfr+/−/− mice occurs via a mechanism involving NO. These results suggest that “pathophysiological senescence” or “stress-induced senescence” involves a defect in NO production. Vasa et al. (31) showed that exogenous NO increased telomerase activity and delayed endothelial senescence. Importantly, our study demonstrates that sepiapterin treatment upregulates telomerase, which elongates telomeres, thereby counteracting telomere length reduction. The demonstration that Mthfr deficiency affects NO production and thus telomerase activity and induces EPC senescence demonstrates a new paradigm contributing to the deleterious effect of HHcy in cardiovascular disease. Indeed, EPC senescence and the consequent reduction of the ability of EPCs to repair the endothelium may explain the prothrombotic and proatherogenic state of HHcy in experimental animals and humans.

Sirtuins (SIRTs) regulate cell senescence and are considered to be longevity factors, based on experimental observations that increased expression of Sir2 orthologs is sufficient to increase life span in lower organisms (11). SIRT1...
exerts protective effects against endothelial dysfunction by preventing H$_2$O$_2$-induced premature senescence. In human umbilical vein endothelial cells, inhibition of SIRT1 decreased eNOS expression and activity (21). Recently, effective antioxidants such as resveratrol have been shown to act by stimulating endothelial SIRT1, which regulates endothelium-dependent vasodilation and bioavailable NO through NOS and stimulates eNOS activity and increases endothelial NO (19). However, NO can also regulate the expression of SIRT1 during aging of endothelial cells (22). Our results demonstrate that uncoupling of eNOS leads to decreased expression of SIRT1, and ultimately to increased stress-induced senescence. More importantly, treatment with sepiapterin improved expression of Tert and SIRT1 and thereby decreased senescence. On the basis of our results, we propose this as a possible signaling pathway in EPCs whereby Mthfr deficiency induces premature senescence.

The mechanism described here could explain why HHcy has been associated with increased cardiovascular risk. Considering that there is a known activator of SIRT1, resveratrol, this might represent an attractive target for drug development. Furthermore, resveratrol not only stimulates SIRT1 (see above) but has also been shown to exert antioxidant properties by inducing the expression of antioxidant enzymes like SOD, glutathione peroxidase, and catalase (29, 30, 43). However, the rather ubiquitous expression and broad effects of SIRT1 might also pose significant hurdles with regard to specificity and side effects. It was recently demonstrated that eNOS plays a crucial role in the functional activity of stem or progenitor cells (1). Activation of eNOS in EPCs may constitute an avenue to improve stem cell therapy in pathological settings associated with progenitor cell dysfunction. Thus we propose here an indirect and probably more specific way to activate SIRT1 by stimulation of eNOS, which improved EPC differentiation and function by protecting EPCs from Mthfr deficiency-induced senescence.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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