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ORIGINAL RESEARCH

# Overexpression of Nitric Oxide Synthase Restores Circulating Angiogenic Cell Function in Patients With Coronary Artery Disease: Implications for Autologous Cell Therapy for Myocardial Infarction

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***Background*—**Circulating angiogenic cells (CACs) are peripheral blood cells whose functional capacity inversely correlates with cardiovascular risk and that have therapeutic benefits in animal models of cardiovascular disease. However, donor age and disease state influence the efficacy of autologous cell therapy. We sought to determine whether age or coronary artery disease (CAD) impairs the therapeutic potential of CACs for myocardial infarction (MI) and whether the use of ex vivo gene therapy to overexpress endothelial nitric oxide (NO) synthase (eNOS) overcomes these defects.

***Methods and Results*—**We recruited 40 volunteers varying by sex, age (< or ≥45 years), and CAD and subjected their CACs to well-established functional tests. Age and CAD were associated with reduced CAC intrinsic migration (but not specific response to vascular endothelial growth factor, adherence of CACs to endothelial tubes, eNOS mRNA and protein levels, and NO production. To determine how CAC function influences therapeutic potential, we injected the 2 most functional and the 2 least functional CAC isolates into mouse hearts post MI. The high-function isolates substantially improved cardiac function, whereas the low-function isolates led to cardiac function only slightly better than vehicle control. Transduction of the worst isolate with eNOS cDNA adenovirus increased NO production, migration, and cardiac function of post-MI mice implanted with the CACs. Transduction of the best isolate with eNOS small interfering RNA adenovirus reduced all of these capabilities.

***Conclusions*—**Age and CAD impair multiple functions of CACs and limit therapeutic potential for the treatment of MI. eNOS gene therapy in CACs from older donors or those with CAD has the potential to improve autologous cell therapy outcomes. **(*J Am Heart Assoc*. 2016;5:e002257 doi: 10.1161/JAHA.115.002257)**

**Key Words:** circulating angiogenic cells • endothelial progenitor cells • gene therapy • myocardial infarction • nitric oxide synthase

Because coronary artery disease (CAD) usually manifests at an advanced age, autologous cell therapy strategies for CAD typically rely on cells isolated from aged, diseased donors. If the age or disease state of the patient adversely affects the therapeutic properties of the otherwise efficacious cells, then it produces a conundrum for successful therapy.[1]

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Circulating angiogenic cells (CACs), also called early endothelial progenitor cells (EPCs), are circulating hematopoietic cells thought to aid in endothelial maintenance and angiogenesis.[2] Circulating cells presumed to be endothelial progenitors were initially identified by expression of endothelial and stem cell markers,[3] and by their functional capacity to facilitate endothelialization of vascular grafts[4] and neovascularization of ischemic tissue.[5] Many subsequent studies considered them to be a single cell type and focused on their functional abilities in culture as prognostic indicators of cardiovascular risk.[6–12] However, it has become apparent that cells derived from the human peripheral blood mononuclear fraction include at least 2 fundamentally distinct populations.[13] The "early" population (appearing after 4 to 7 days in culture) is currently thought to consist of hematopoietic cells similar to monocytes that home to sites of angiogenesis or vascular damage and produce angiogenic signals that help the endothelium grow or regenerate.[2,14] Some of these angiogenic factors may be artifacts of cell isolation,[15] but others are endogenously produced.[16] While some of our earlier publications referred to these cells as EPCs,[17,18] because they do not appear to differentiate into endothelial cells, various groups, including our own, currently refer to these cells as CACs.[2]

Transplantation of CACs or phenotypically similar cells from bone marrow has been investigated as a therapy for a variety of ischemic diseases in animal models.[5,19,20] Results from a preliminary non–placebo-controlled clinical trial of autologous CAC delivery after acute myocardial infarction (MI) suggest that CACs may reduce deleterious cardiac remodeling and improve outcomes,[21,22] although this therapeutic effect did not extend to chronic heart failure.[23] A potential problem is that functional properties of human CACs in culture, which are correlated with the health status of the donor,[6–8,16,24] are also a major determinant of posttransplantation outcome.[21] Thus, the potential therapeutic benefit of autologous CAC infusion to treat cardiovascular disease is likely to be impaired in the patients who might benefit the most from this approach. This is a problem for cell therapy in general, as we and others have shown that autologous bone marrow cell therapy can be thwarted if the subject's age or disease impairs the therapeutic functionality of the cells in question.[25–28] It follows that improving specific functional properties may enhance the therapeutic potential of CACs for the treatment of cardiovascular disease.

Several strategies have attempted to improve therapeutic potential of CACs or related cells. These have included adenoviral transduction of healthy human CACs to express vascular endothelial growth factor (VEGF), which improved their ability to restore function in a mouse model of hindlimb ischemia[29]; and pretreatment of CACs with osteopontin, which increased the diameter of neovessels formed in CAC-seeded collagen plugs during wound healing.[30] More recent strategies have focused on identifying disease-related deficiencies in the expression of factors that are crucial for CAC function and on treating patient-derived cells to overexpress such factors, with the goal of restoring their therapeutic potential for ex vivo expansion and autologous reintroduction.

One promising target is endothelial nitric oxide (NO) synthase (eNOS), which produces NO, a major endothelial mediator regulating vascular homeostasis and modulating arterial tone. NO also modulates angiogenesis in response to tissue ischemia[31,32] and specifically mediates the migration of cultured endothelial cells.[33] CACs express eNOS,[3,34] which plays an important role in their function and in vivo mobilization.[16,18,24,35–38] Mobilization of CACs is impaired in eNOS-deficient mice.[35,37] We have reported that CACs isolated from CAD patients have substantially reduced levels of eNOS protein and are impaired in both random migration and chemotaxis toward VEGF, but migratory response to exogenous NO sources is not impaired.[16] Thus, eNOS and NO appear to play a critical role in CAC function, and eNOS expression is a promising target for enhancement.

Sasaki et al[38] reported that transient, pharmacologically induced transcriptional activation of eNOS in CACs results in increased perfusion potential in a mouse hindlimb ischemia model. While this is presumably a temporary effect, Zhao et al[39] and Ward et al[24] showed that transduction of CACs to stably overexpress eNOS enhances their therapeutic effects in a rat model of pulmonary hypertension and in mouse ischemic hindlimb, and Kong et al[40] showed that eNOS-transduced CACs inhibit intimal hyperplasia in injured vessels.

We hypothesized that for optimal CAC-based therapy for post-MI remodeling, the varying therapeutic potential of CACs from different donors would benefit from modulation of eNOS expression in dysfunctional cells. We report here that in CACs isolated from 40 human subjects, age and CAD drive impairments of multiple functional properties that decrease NO production and reduce therapeutic efficacy of CAC implantation into mouse hearts post MI. We further show that poor therapeutic efficacy of CAD patient CACs is substantially improved by eNOS ex vivo gene therapy and that high therapeutic efficacy of young healthy donor CACs is similarly reduced by eNOS knock-down.

## Methods

### Subject Characteristics

CACs were isolated from 10 participants in each of the following 4 categories: healthy women aged <45 years, healthy men aged <45 years, healthy men and women aged ≥45 years, and men and women aged ≥45 years with documented CAD.

Inclusion criteria for healthy participants included being 18 years or older and the absence of hypertension (blood pressure >140/90 mm Hg), dyslipidemia (low-density lipoprotein >160 mg/dL), diabetes mellitus (fasting plasma glucose >126 mg/dL), cigarette smoking, evidence of coronary or peripheral artery disease, malignancies, terminal renal failure, acute inflammation, pregnancy, medication with statins, estrogen replacement, hormonal birth control, or erectile dysfunction medication. The young, healthy female participants underwent flow-mediated dilation (FMD) measurement and blood draws within 3 days of the start of the menstrual cycle to minimize hormonal variability. The CAD group consisted of 10 patients recruited through the University of California, San Francisco (UCSF) Cardiology Clinic with angiographically documented CAD as defined by >70% stenosis of ≥1 coronary artery, receiving optimal medical therapy according to current secondary prevention guidelines.[41] Informed consent was obtained in accordance with the UCSF Institutional Review Board and the Declaration of Helsinki. Subject characteristics of all participants with complete data are described in Table 1.

## FMD Measurement

Endothelial function determined by FMD of the brachial artery was measured by using ultrasonography (Sonosite Micromax). Subjects rested in the supine posture in a room at 20° to 22° C for at least 10 minutes, fasted for ≥12 hours before the measurement, and refrained from caffeine and alcohol intake and exercise for ≥8 hours before the measurement. A cuff was placed distal to the probe and was inflated 250 mm Hg for 5 minutes. Diameter was measured before cuff inflation and immediately after cuff deflation, at 20, 40, 60, and 80 seconds. FMD was calculated as Percent change=(Peak $\text{diameter}_{\text{postischemia}} - \text{Diameter}_{\text{baseline}})/\text{Diameter}_{\text{baseline}} \times 100$. All measurements were performed by the same examiner. Videos were analyzed offline by an automated analysis system (Brachial Analyzer, Medical Imaging Applications) as described earlier.[17] Measurement of endothelium-independent vasodilation, sometimes assessed in FMD studies by the administration of nitroglycerin, was not approved by the institutional review board and therefore was not attempted.

**Table 1.** Baseline Characteristics of Subjects in the Study

| Characteristic | Group | | | |
|---|---|---|---|---|
| | Healthy Young Women Aged <45 y | Healthy Young Men Aged <45 y | Healthy Older Patients Aged ≥45 y | CAD Patients Aged ≥45 y |
| No. (M/F) | 10 (0/10) | 10 (10/0) | 10 (6/4) | 10 (7/3) |
| Age, y | 28.2±1.7 | 28.0±2.2 | 51.1±1.5* | 71.5±2.6*† |
| Body mass index, kg/m$^2$ | 21.3±0.7 | 24.0±0.7 | 26.4±1.3‡ | 27.2±1.2c |
| Systolic blood pressure, mm Hg | 96.3±1.6 | 114.0±2.9§ | 118.7±3.9‡ | 140.8±5.8*¶ |
| Diastolic blood pressure, mm Hg | 61.1±1.9 | 66.4±2.5 | 70.8±3.3§ | 69.7±1.3 |
| Fasting glucose, mg/dL | 89.9±2.0 (n=9) | 99.1±5.5 | 95.0±2.6 | 90.2±8.0 |
| Total cholesterol, mg/dL | 160.1±8.6 (n=9) | 159.9±6.5 | 178.6±7.4 | 141.3±9.0# (n=9) |
| LDL cholesterol, mg/dL | 83.6±8.8 (n=9) | 96.2±7.3 | 110.8±4.3 | 72.4±7.3¶ (n=8) |
| HDL cholesterol, mg/dL | 63.2±4.1 (n=9) | 54.1±4.1 | 57.2±6.6 | 44.8±4.2§ (n=9) |
| Triglycerides, mg/dL | 66.4±13.8 (n=9) | 48.3±6.0 | 77.2±14.4 | 139.2±36.0** (n=9) |
| ACE inhibitor | 0% | 0% | 0% | 30% |
| Angiotensin type II receptor blocker | 0% | 0% | 0% | 30% |
| Diuretics | 0% | 0% | 0% | 50% |
| Clopidogrel | 0% | 0% | 0% | 60% |
| Statins | 0% | 0% | 0% | 100% |
| β-Blockers | 0% | 0% | 0% | 100% |
| Aspirin | 0% | 0% | 0% | 100% |

Data given as mean±SEM. For each parameter, n=10/group unless otherwise stated. ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*$P$<0.001 compared with both healthy young groups.
†$P$<0.001 compared with healthy older group.
‡$P$<0.01 compared with healthy young female group.
§$P$<0.05 compared with healthy young female group.
¶$P$<0.01 compared with healthy older group.
#$P$<0.05 compared with healthy older group.
**$P$<0.01 compared with healthy young male group.

## Collection and Characterization of Peripheral Blood CACs

Blood samples (13 mL) were collected from the cubital vein on the morning of the ultrasound examination after a 12-hours overnight fast to measure the serum lipid profile and other biochemical parameters. In a second visit that did not involve fasting, 250 mL of blood was collected for CAC isolation into Vacutainer CPT tubes (Becton Dickinson) with heparin. CACs were differentiated ex vivo from peripheral blood mononuclear cells by 7 days of culture as previously described.[2,7] Peripheral blood mononuclear cells were isolated through density centrifugation in Accuspin™ System-Histopaque-1077 (Sigma-Aldrich) and preplated on fibronectin-coated dishes in endothelial cell basal medium (EBM-2; Cambrex) supplemented with EBM-2MV SingleQuot and 20% fetal bovine serum for 3 hours to remove platelets and shed endothelial cells, which were discarded. The initially nonadherent cells were harvested and frozen at a concentration of $1\times10^7$ cells/mL in EBM-2 with EBM-2MV SingleQuot, 20% fetal bovine serum, and 10% DMSO for later use. Before subsequent experiments, the peripheral blood mononuclear cells were thawed and cultured on fibronectin-coated dishes for 7 days as before.

Because of the large number of CAC donors and assays, it was not feasible to count postculture CACs that were positive for standard markers. However, in 3 of our previous studies,[16–18] we isolated human CACs from small groups of donors and reported that after 7 days of culture, >95% of them were double-positive for *Ulex europeus* agglutinin lectin binding and acetylated low-density lipoprotein uptake. In 2 of the studies,[16,18] we reported that the cell populations expressed CD45 (85–93%), KDR (24–49%), CD31 (49–63%), CXCR4 (57–59%), CD14 (68–70%), and CD11b (54–55%); with extremely low incidence (≈1%) of postculture cells expressing CD34 or CD133. CACs isolated from healthy young volunteers and older CAD patients in our earlier pilot study[16] did not differ significantly in expression of CD45 or CD31 postculture.

## Migration Assay

Migration of CACs was quantified with a transwell chemotaxis assay by using a modified Boyden chamber. Per our previously published protocol,[18] 600 μL of EBM-2 media with or without 50 ng/mL VEGF (Sigma) was added to the bottom of a 24-well transwell chamber plate (Corning). Then, $2\times10^4$ CACs (post 7-day culture) were resuspended in 100 μL EBM-2 supplemented with 0.5% bovine serum albumin, added to each migration insert (8-μm pores; Corning), and placed in the companion 24-well tissue culture plate. Each sample was loaded in triplicate inserts. Cell migration occurred during a 6-hours incubation at 37°C. Cells attached to the underside of the insert membrane were fixed in 4% formaldehyde, and cells attached to the topside of the membrane were removed with a cotton swab. The membrane was removed, mounted on a glass slide, and stained by using Hoechst 33342. Fluorescence microscopy was used to capture 5 random fields (×10 objective) per membrane, and results were expressed as the average of the number of cells visualized per field.

## Assays for CAC Adhesion to Human Umbilical Vein Endothelial Cells and Association With Human Umbilical Vein Endothelial Cell Tubes

CAC adhesion to endothelial cells and association with endothelial tubules were performed as previously described.[9] For adhesion, $2\times10^5$ human umbilical vein endothelial cells (HUVECs) at passages 3 to 5 were seeded per well in 4-well glass slides. At 36 hours later, some wells were pretreated with 1 ng/mL tumor necrosis factor-α (BD Biosciences) for 12 hours; other wells were not pretreated. Post–7-day CACs in suspension were incubated with Vybrant® Dil Cell-Labeling Solution (2.5 μg/mL in PBS; Life Technologies) for 5 minutes at 37°C followed by 15 minutes at 4°C and then were added to the wells containing HUVEC monolayers and incubated for 3 hours. After the nonattached cells were washed out with PBS, the HUVECs and adhered CACs were fixed with 4% paraformaldehyde and counted in 10 random fields.

For the HUVEC tube-forming assay, 4-well glass slides coated with Matrigel (BD Bioscience) were put in the incubator at 37°C for 30 minutes to allow solidification. Dil-labeled CACs ($2\times10^4$) were coplated with $4\times10^4$ HUVECs and incubated at 37°C for 6 hours to allow the HUVECs to form tubes. The percentage of CACs associated with tubes[9] was determined in 10 random fields by counting the number of stained cells colocalizing with tubes and dividing by the total number of stained cells, and then multiplying by 100.

## Real-Time Reverse-Transcription–Polymerase Chain Reaction Analysis

Total RNA from cultured CACs was isolated by using an RNeasy Mini Kit (Qiagen) or RNAqueous-Micro Kit (Ambion). cDNA was transcribed from RNA with iScript cDNA Synthesis Kit (Bio-Rad). Real-time polymerase chain reaction (PCR) was then performed on a sequence-detection system (Prism 7900; Applied Biosystems) and the TaqMan PCR Core Reagent kit (Applied Biosystems). β-Actin was used as the endogenous control to normalize the amount of cDNA added to each reaction for analysis of eNOS expression; primers were purchased from Applied Biosystems. Human β-glucuronidase was the endogenous control for CD14, KDR, CD31, and CD45 expression; primers were purchased from Integrated DNA Technologies. PCR was performed at the Genome Analysis Core Facility, Helen Diller Family Comprehensive Cancer Center, UCSF.

## Immunoblotting and ELISA

eNOS level in undiluted cell lysate was quantified by the use of ELISA (R&D Systems) in samples prepared from $1\times10^6$ cultured CACs in 1 mL of lysis buffer from the manufacturer. Each sample was assayed in duplicate. To normalize the eNOS protein levels, total protein concentrations were measured by using Coomassie Plus–The Better Bradford™ Assay Kit (Pierce). eNOS in Western blots was detected by using eNOS/NOS Type III antibody (1:1000, BD Transduction Laboratories) followed by corresponding IRDye 800CW goat anti-mouse second antibody (1:5000; LI-COR Biosciences) and visualized and quantified by using an Odyssey® infrared imaging system (LI-COR).

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex, cultured in EBM-2 (supplemented with Singlequots 5% FBS) and used no later than passage 5 as positive controls for eNOS immunoblots.

## Measurement of Nitrate/Nitrite Level in Cell Culture Medium

Thawed peripheral blood mononuclear cells were seeded in a 6-well plate and, at day 6, washed with phosphate-buffered saline; the medium was replaced with 1 mL fresh culture medium and incubated overnight. At day 7, medium was collected and frozen at −80°C for measurement of liberated NO. NO levels were evaluated by measuring the intermediate and end products, nitrate and nitrite ($NO_x$). $NO_x$ was reduced by using vanadium (III) and hydrochloric acid at 90°C. NO was purged from the solution, resulting in a peak of NO for subsequent detection by chemiluminescence (NOA 280; Sievers Instruments). The emitted light signal was recorded by dedicated software as the NO content (micromoles per liter).

Stimulation of eNOS was achieved by treatment of CACs with 10 μmol/L ionomycin for 2 hours (Sigma-Aldrich).

## Adenoviral Transduction

Adenoviruses Ad.CMV-eNOS and Ad.CMV-GFP (green fluorescent protein) (S.M. Black, PhD, unpublished) and Ad-eNOS-siRNA (gift from David Fulton[42]) were used to transduce CACs. CACs were infected at day 5 of culture with Ad.CMV-GFP, Ad.CMV-eNOS, or Ad-eNOS-siRNA at a multiplicity of infection of 100 or 500. After overnight incubation, the cells were washed twice with phosphate-buffered saline, fresh culture medium was added, and cells were used for the relevant experiments at day 7 of culture.

## Animals

All animal procedures were approved by the UCSF Institutional Animal Care and Use Committee and performed in accordance with the recommendations of the American Association Accreditation of Laboratory Animal Care. SCID mice (C.B-17, male, 10 weeks) were obtained from Taconic. Group size ranged from 6 to 10 depending on the number of individual donor CACs available; vehicle control group was n=5.

## Surgical Induction of MI

MI was surgically induced through permanent coronary artery ligation as we have described previously.[43] Briefly, mice were anesthetized with 2% isoflurane during surgery and received subcutaneous buprenorphine (0.1 mg/kg) for analgesia at the time of surgery and at the end of the day. The heart was exposed via parasternotomy, and the left anterior descending coronary artery was permanently ligated ≈3 mm below the tip of the left atrium.

## CAC Injection Into Mouse Hearts Post MI and Analysis of Cardiac Function and Infarct Size

End-diastolic and end-systolic volumes and left ventricular ejection fraction (LVEF) were assessed by using echocardiography with 1.25% isoflurane (Vevo660 micro-ultrasound system; VisualSonics Inc) as we have previously described.[27,44] For CAC injections into myocardium, $10^5$ viable CACs in Hanks balanced salt solution (HBSS) were split into 2 injections of 5 μL and injected into myocardium at the infarct border zone with use of a 30-gauge needle under ultrasound visualization with 2% isoflurane at 3 days post-MI, according to our previously published procedure.[43] Echocardiography was performed and interpreted by a blinded investigator. Infarct size was calculated through histological measurement of the fibrotic zone according to our published protocol.[45]

## Immunofluorescence Staining

Mice were killed at day 28 post-MI/day 25 postimplantation, and hearts were frozen in freezing isopentane and cryosectioned at 10 μm. Sections were fixed in 1.5% formaldehyde for 15 minutes and then blocked with normal goat serum staining buffer for 30 minutes without detergent (2% normal goat serum and 0.02% sodium azide in phosphate-buffered saline). For quantitation of blood vessels and CACs, sections were further blocked with use of the Avidin and Biotin Blocking System (DAKO) each for 10 minutes. Slides were then incubated in primary antibody for 1 hour, consisting of rat anti–human leukocyte antigen (HLA) monoclonal antibody (Bio-Rad; 1:400) and mouse anti–smooth muscle actin monoclonal antibody (clone 1A4; ICN MP Biomedicals; 1:400), as well as biotin conjugated BS-1 isolectin B4 (Sigma-Aldrich, 1:100). Negative controls lacking primary antibody/lectin were always performed. Sections were rinsed

in staining buffer and then incubated for 1 hour at room temperature with Alexa 488 goat anti-rat antibody (1:250), Alexa 546 goat anti-mouse antibody (1:200), or Alexa 660 streptavidin (1:100; secondary antibodies from BD Pharmingen). Alternatively, for initial detection of CACs 3 days after implantation, CACs had been stained with cytoplasmic Cell Tracker Green (Life Technologies) according to the manufacturer's instructions before implantation. These sections were stained as described here earlier only with HLA antibody. The slides in all cases were then rinsed, mounted, and viewed with a Nikon E800 fluorescence microscope by using Openlab software (Improvision).

For quantitation of capillary density represented by total endothelial stain, photomicrographs were taken with a ×20 objective, and endothelium-stained regions were automatically selected by using ImagePro software (Media Cybernetics), normalized for regions of visual fields lacking tissue and expressed as percentage of the field that stained positive for endothelium. For quantitation of arterioles and venules, the number of structures in the same photomicrographs staining positive for smooth muscle actin were counted, normalized for regions of visual fields lacking tissue, and expressed as number of objects per field.

## Statistical Analyses

For in vitro/ex vivo experiments, data are presented as mean±SEM. Planned comparisons central to the research design were tested by using a family-wise $\alpha$ of 0.05 as specific contrasts within an ANOVA test for each biological outcome, with group status as the independent variable (with the use of SPSS software). The a priori hypotheses were that FMD and CAC function would be greatest in young men and women, significantly decreased in healthy older individuals, and significantly further decreased in CAD patients. Because we did not make specific hypotheses about differences between younger men and women, we used a more stringent critical $\alpha$ of 0.025 when comparing each of the younger groups separately (men or women only) with the older groups. For all other pairwise differences reported, Tukey post hoc corrections were used. We used Pearson correlations to investigate associations between functional properties of the groups.

For mouse cardiac functional experiments, data are presented as mean±SD. To compare means between multiple (>2) groups on days 2 and 28 post MI, SPSS was used to apply 2-way repeated-measures ANOVA, and pairwise significance was determined with Tukey post hoc correction. An interaction between group and time was included in the model. To compare means between day 2 and day 28 post MI in each group, 2-tailed paired t test was applied. $P<0.05$ regarded as significant.

# Results

## Characteristics of Human Subjects

Characteristics of the study subjects are shown in Table 1. Systolic blood pressure showed increases associated with male sex, age, and CAD. However, diastolic blood pressure in the CAD group was comparable to that of the healthy older group and was not significantly higher than that in the younger groups. The CAD group, in which all patients were being treated with statins, exhibited lower total cholesterol and low-density lipoprotein than the older healthy group. Notably, these conditions did not prevent brachial artery FMD, a measure of endothelial function, from being significantly lower in the CAD group than in both young healthy groups and the aged healthy group (Figure 1).

## Intrinsic Migratory Ability, Rather Than Chemotaxis Toward VEGF, is Impaired in CAD CACs

Migration of CACs toward VEGF has repeatedly been shown to correlate with cardiovascular health of the donor.[6,8,16,24] As shown in Figure 2A, the CACs from the young male group migrated toward VEGF more than did those from the CAD group (24.4±2.9 versus 10.5±1.3 per field, $P=0.001$), as did those from the young female group (19.3±2.9 versus 10.5±1.3, $P=0.022$; n=10/group for all migration experiments). Notably, as shown in Figure 2B, random migration in the absence of a VEGF gradient was also higher in young male CACs than in CAD CACs (12.3±1.6 versus 6.9±0.9, $P=0.007$) and higher in young female CACs than in CAD CACs

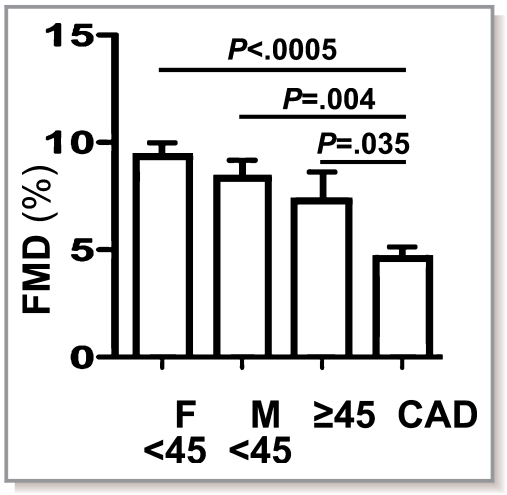


**Figure 1.** Flow-mediated dilation (FMD) was impaired in coronary artery disease (CAD) patients. Groups shown are young healthy women, young healthy men, older healthy subjects, and CAD patients. n=10/group.

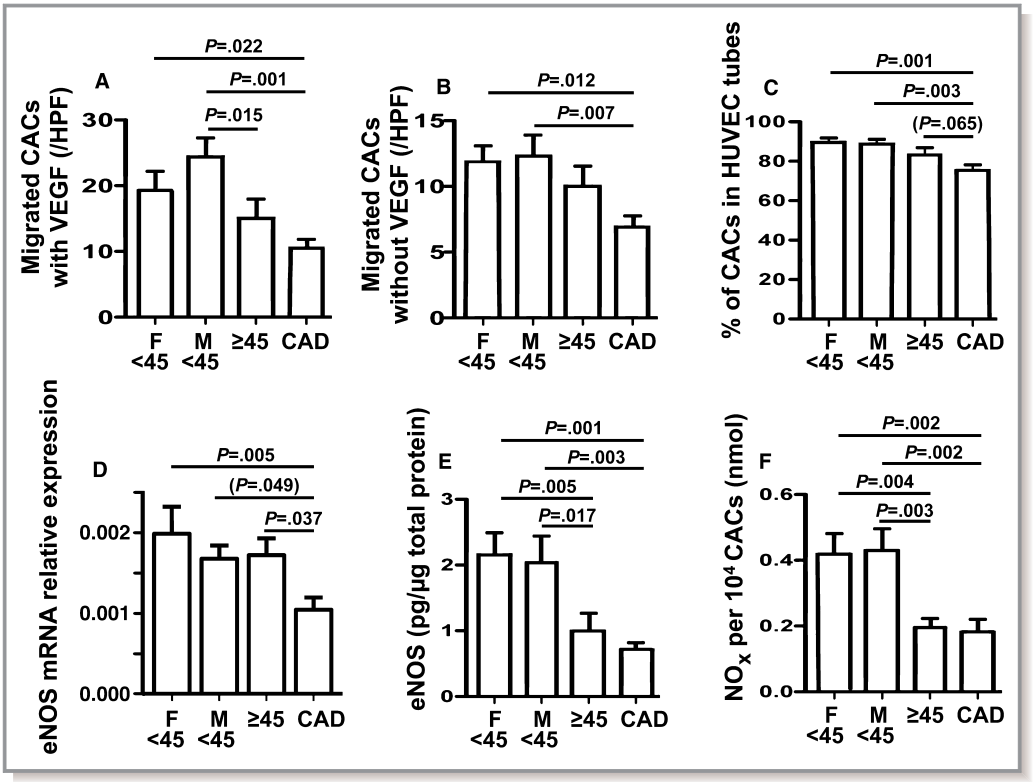


**Figure 2.** Advanced age and coronary artery disease (CAD) each influence different functional properties of circulating angiogenic cells (CACs). A and B, CAD-related decline in both migration toward a vascular endothelial growth factor (VEGF) gradient and random movement in the absence of VEGF. C, CAD-related decline in CAC association with endothelial tubes. D, CAD-related decline in endothelial nitric oxide synthase (eNOS) mRNA levels, graphed as percent expression of eNOS relative to β-actin in the 4 CAC groups. One high data point, in the young healthy female group, was Winsorized. E and F, Age-related decline in eNOS protein levels and in nitric oxide (NO) production as determined by nitrate and nitrite ($NO_x$) measurement of conditioned medium. n=7 to 10/group as described in the Results section.

(11.9±1.2 versus 6.9±0.9, *P*=0.012). In fact, when migration toward VEGF was normalized to random migration, no relationship achieved significance (not shown), demonstrating that intrinsic migration, rather than specific chemotactic response toward VEGF, is impaired in CACs from CAD patients.

Migration toward VEGF was significantly higher in the young male group compared with healthy older adults (24.4±2.9 versus 15.00±3.0 per field, *P*=0.015), but none of the other pairwise comparisons for VEGF or control migration approached significance (*P*>0.1 for all).

## Association of CACs With Cocultured Endothelial Tubes is Impaired in CAD CACs

While late-outgrowth EPCs have been reported to differentiate ex vivo into capillary-like tubes, CACs by themselves do not form tubes.[2] However, CACs cocultured with endothelial cells will adhere to the endothelial tubes (Figure 3), a property that is decreased in CACs from diabetic subjects who exhibit other functional deficits.[9] This property may reflect the homing of CACs to angiogenic endothelium and their association with the endothelial layer. When cocultured with HUVECs, association of CACs with tubes was slightly but significantly higher in the young healthy women (n=10) and men (n=9) relative to the CAD group (n=9) (89.6±2.3% and 88.8±2.4% versus 75.35±2.9%, *P*=0.001 and 0.003, respectively) but not relative to that of the older healthy group (83.14±3.7%; n=10, both *P*s>0.1) (Figure 2C). Association of CACs was higher in the older healthy group than in the CAD group with a trend toward significance (*P*=.065; critical α was 0.05 for this relationship). We did not observe group-specific pattern differences in the associated tubes (not shown).

We also examined whether age or CAD would reduce the number of CACs to adhere to quiescent or tumor necrosis factor-α–activated endothelial monolayers, as described by Tepper et al[9] for CACs from diabetic patients. We did not observe differences between groups in adhesion to monolayers (not shown), in agreement with Ward et al.[24]

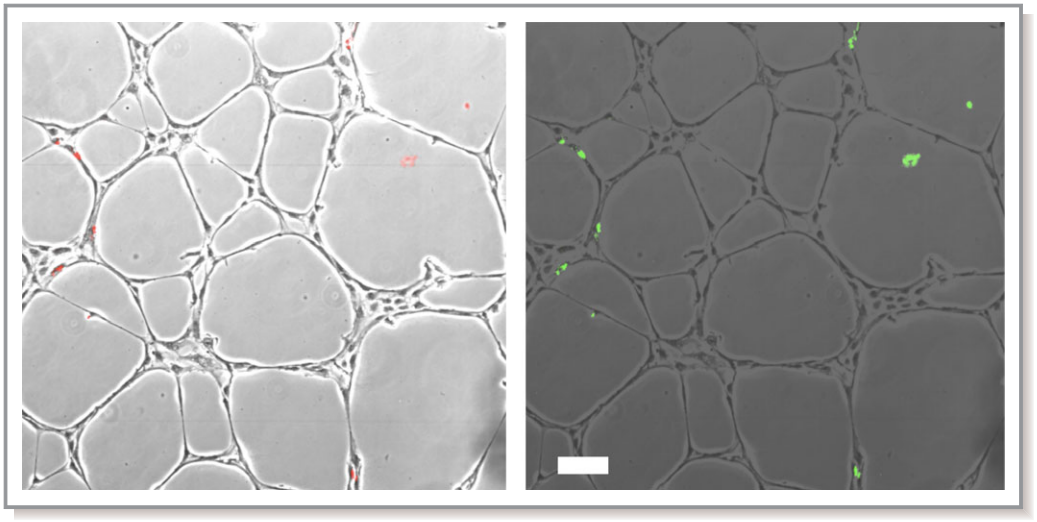

**Figure 3.** Example of circulating angiogenic cell (CAC) association with human umbilical vein endothelial cell (HUVEC) tubes. Left: CACs labeled with DiI (red) are shown within the HUVEC tube (phase contrast optics). Right: Enhanced, image-processed version in which the phase contrast image has been darkened and flattened, and the red has been pseudo-colored green for contrast. Bar=100 μm.

## The Aged Healthy and CAD Groups Demonstrated Separate Levels of eNOS Regulation

To determine how age and CAD influence production of NO from CACs, we measured both eNOS mRNA and eNOS protein in CAC lysates and measured nitrite in the culture medium as an indication of NO release from the cells. Figure 2D shows real-time RT-PCR results for eNOS in the CAC groups. The eNOS RNA level in the CAD group was significantly decreased relative to the young healthy female group (0.0010±0.0001 versus 0.0019±0.0003 relative unit, $P$=0.005; n=10 for all CAC groups). The differences between the CAD and young healthy male group (0.0017±0.0001) approached but did not reach significance ($P$=0.049; critical α was considered to be 0.025 for this relationship), whereas the similar difference between the older healthy (0.0017±0.0002) and CAD groups achieved significance with $P$=0.037 because the critical α was 0.05 (see Statistical Analyses). These results, along with the lack of a decrease in eNOS mRNA in the aged healthy group relative to either younger group (no difference approached significance), suggest that CAD was the driving effect. However, because the average age of the CAD group was greater than that of the aged healthy group, it is unclear whether this effect was driven by CAD per se or by even greater age (see Discussion).

In contrast, eNOS protein level measured with ELISA as picogram per microgram total cellular protein (Figure 2E) was higher in both healthy young women (2.2±0.3; n=9) and healthy young men (2.0±0.4; n=7) than in the CAD group (0.70±0.1; $P$=0.001 and 0.003, respectively) and the healthy older group (1.0±0.3; $P$=0.005 and 0.017) (each n=10). The interpretation of age as the driving factor rather than CAD is bolstered by lack of difference between the aged healthy and CAD groups ($P$=0.46). Further, $NO_x$ (reflecting NO release) in conditioned culture media from the CACs (Figure 2F) showed an age-related effect with almost identical values in the older healthy (0.20±0.03 nmol/$10^4$ CACs) and CAD (0.18±0.04 nmol/$10^4$ CACs) groups (both n=10) that were significantly lower than those in the healthy young female (0.40±0.06 nmol/$10^4$ CACs; n=9) and healthy young male (0.43±0.07 nmol/$10^4$ CACs; n=7, all $P$≤0.004 for all) groups.

In a separate follow-up experiment, CAD CACs were still able to increase NO generation when stimulated with ionomycin (9.2±3.8 nmol/$10^4$ unstimulated CACs versus. 13.5±5.3 nmol/$10^4$ stimulated CACs; $P$=0.0003 by 1-tailed paired t test).

## Positive Correlation Between CAC Migration and eNOS Protein Level

A Pearson correlation matrix revealed similar correlations or lack thereof across the whole population (detailed results are shown in Table 2). CAC migration, dependent on intrinsic motility rather than chemotaxis, correlated significantly with eNOS protein levels ($r$=0.44, $P$≤0.01) with a borderline significant correlation with eNOS mRNA ($r$=0.27, $P$≤0.07). CAC migration also correlated with FMD ($r$=0.49, $P$≤0.01). However, the correlation between FMD and eNOS mRNA ($r$=0.20) or protein ($r$=0.15) did not reach significance, suggesting that of the outcomes analyzed, migration is the

**Table 2.** Pearson Correlation Matrix Showing Correlations Between the Parameters Studied

| Correlation: *r* (n) | eNOS (qPCR) | eNOS (Protein) | Nitrate (Medium) | FMD | Migration (C) | Migration (V) | Migration (V/C) | Sysolic BP | Percent in Tubes | Adhesion |
|---|---|---|---|---|---|---|---|---|---|---|
| eNOS (qPCR) | — | 0.30 (36) | 0.17 (40) | 0.20 (40) | 0.27* (40) | 0.26 (40) | 0.09 (40) | −0.35† (40) | 0.30 (38) | −0.09 (40) |
| eNOS (protein) | 0.30 (36) | — | 0.32* (36) | 0.15 (36) | 0.44‡ (36) | 0.26 (36) | −0.06 (36) | −0.37† (36) | 0.27 (34) | −0.13 (36) |
| Nitrate (medium) | 0.17 (40) | 0.32* (36) | — | 0.26 (40) | 0.13 (40) | 0.30* (40) | 0.11 (40) | −0.36† (40) | 0.32* (38) | 0.04 (40) |
| FMD | 0.20 (40) | 0.15 (36) | 0.26 (40) | — | 0.41‡ (40) | 0.40† (40) | 0.04 (40) | −0.41‡ (40) | 0.29 (38) | 0.22 (40) |
| Migration (C) | 0.27* (40) | 0.44‡ (36) | 0.13 (40) | 0.41‡ (40) | — | 0.68§ (40) | −0.14 (40) | −0.36† (40) | 0.00 (38) | −0.24 (40) |
| Migration (V) | 0.26 (40) | 0.26 (36) | 0.30* (40) | 0.40† (40) | 0.68§ (40) | — | 0.54§ (40) | −0.34† (40) | 0.06 (38) | −0.24 (40) |
| Migration V/C | 0.09 (40) | −0.06 (36) | 0.11 (40) | 0.04 (40) | −0.14 (40) | 0.54§ (40) | — | 0.04 (40) | 0.06 (38) | −0.16 (40) |
| Systolic BP | −0.35† (40) | −0.37† (36) | −0.36† (40) | −0.41‡ (40) | −0.36† (40) | −0.34† (40) | 0.04 (40) | — | −0.43 ‡ (38) | −0.01 (40) |
| Percent in tubes | 0.30 (38) | 0.27 (34) | 0.32† (38) | 0.29 (38) | 0.00 (38) | 0.06 (38) | 0.06 (38) | −0.43‡ (38) | — | −0.04 (38) |
| Adhesion | −0.09 (40) | −0.13 (36) | 0.04 (40) | 0.22 (40) | −0.24 (40) | −0.24 (40) | −0.16 (40) | −0.01 (40) | −0.04 (38) | — |

Each cell of the table shows correlation *r* value between 2 measurements of certain number (n) of subjects. Adhesion indicates adhesion of CACs to tumor necrosis factor–activated endothelium; eNOS (protein), eNOS protein level measured by ELISA; eNOS qPCR, eNOS transcript level measured by real-time RT-PCR; FMD, flow-mediated dilation in the CAC donors; Migration (C), random migration of CACs; Migration (V), migration to vascular endothelial growth factor (VEGF); Migration V/C, migration to VEGF normalized to random migration; nitrate, nitrate level in circulating angiogenic cell (CAC) culture medium; Percent in tubes, percent of CACs in cocultured human umbilical vascular endothelial cell tubes; Systolic BP, systolic blood pressure of CAC donors at rest.
*$P \leq 0.07$, †$P \leq 0.05$, ‡$P \leq 0.01$, §$P \leq 0.001$. For endothelial nitric oxide synthase (eNOS) qPCR, 1 outlier is Winsorized.

most predictive of overall function. This finding is in agreement with Britten et al,[21] except that intrinsic motility appears to be more important than specific chemotaxis.

## Phenotypic Shift From Endothelial to Monocytic Gene Expression in CAD CACs

In a preliminary exploration of phenotypic changes in the CAD CACs that might underlie their poorer functional performance relative to CACs from the other groups, we assessed changes in expression of representative genes indicative of monocytic or endothelial characteristics, by using real-time RT-PCR analysis of pooled transcripts from each CAC group. Because we had observed only minor differences between young women and young men in our other functional characterizations of the individual CAC isolates, we combined them into a single young donor pool, yielding a total of 3 pools (aged <45 years, aged ≥45 years, and CAD patients). CAC isolates were grown individually in culture so that no single isolate would predominate the RNA complement as a result of faster growth, and the resulting unbiased RNA pools from the 3 groups were analyzed for relative expression of KDR, CD31, CD14, and CD45, normalized to the aged <45 years group (Figure 4). KDR was not detectable. However, while there was not a major shift in CD45 or CD31 expression among the groups, the CAD group had almost double the expression of CD14 of that of the other groups. CD31 went down slightly in that group. Because this experiment was hypothesis generating and involved pools of donor CACs rather than distinct values per donor, no statistical analysis was performed. The pooled approach makes it unclear whether this was because of an increase in CD14 expression by CAD CACs or the results of a larger percentage of $CD14^+$ cells in this heterogeneous sample; this is a launching point for further study.

## Strong Correlation Between CAC Donor Cardiovascular Health, CAC Ex Vivo Functional Level, and Therapeutic Effect of CAC Implantation Into Post-MI Mouse Hearts

We ranked all 40 CAC isolates on the basis of migration and eNOS transcription, and selected 2 of the highest-function

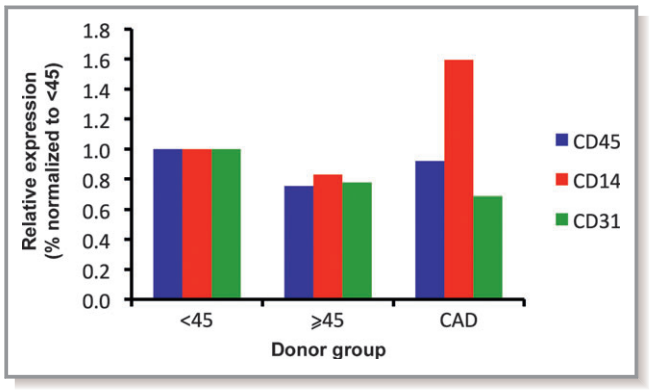


**Figure 4.** Changes in endothelial and hematopoietic marker gene expression showing a shift toward CD14 prevalence in the coronary artery disease (CAD) circulating angiogenic cells. mRNA prevalence in pooled samples from the combined male/female aged <45 years groups, the aged ≥45 years groups, and the CAD group. Each pool consists of 7 to 10 individual donors, but single pools are compared; therefore, no error bars are included.

**Table 3.** Characteristics of CACs Selected for Intramyocardial Implantation and Their Donors

| Characteristic | CAC Isolate | | | |
|---|---|---|---|---|
| | Healthy #1 | Healthy #2 | CAD #1 | CAD #2 |
| Age, y | 26 | 32 | 63 | 67 |
| Sex | Female | Female | Male | Male |
| FMD, % | 11.8 | 9.9 | 7.4 | 2.3 |
| eNOS mRNA, relative units | 24 | 20 | 11 | 4 |
| Migration—control, average No. migrated cells±SEM | 16±2 | 20±3 | 7±1 | 8±0.6 |
| Migration—VEGF, average No. migrated cells±SEM | 31±2 | 30±3 | 9±1 | 10±1 |

CAC indicates circulating angiogenic cell; FMD, flow-mediated dilation; VEGF, vascular endothelial growth factor.

isolates (both from young healthy females) and 2 of the lowest-function isolates (both from CAD patients) for further in vivo experiments. Characteristics of the CACs and their donors are provided in Table 3.

To test our hypothesis that CAC ex vivo function and donor health status would positively correlate with therapeutic potential for MI, we implanted each of these 4 isolates into hearts of separate groups of immunodeficient SCID mice at 3 days after surgical induction of MI. Injection of vehicle (Hanks balanced salt solution [HBSS]) served as a negative control. Closed-chest injection was targeted to the per-infarct border zone of the left ventricular myocardial wall by microultrasound guidance by using a method that we have previously reported,[43] and that we have used successfully to demonstrate therapeutic effects of nonselected bone marrow cells to improve post-MI left ventricular function.[26,27,44,46] As shown in Figure 5A with numerical details in Table 4, the LVEF in all groups significantly decreased on day 2 post MI ($P<0.001$), the day before therapy. From day 2 to day 28 post MI, LVEF in negative control hearts injected with HBSS continued to deteriorate ($P<0.05$). In contrast, LVEF improved ($P<0.05$) from day 2 to day 28 in mice injected with the 2 most functional donor-specific CAC isolates. The 2 least-functional donor-specific isolates either only partially preserved LVEF ($P$=NS) or did not preserve LVEF ($P<0.05$). On day 28, overall comparison between the 5 recipient mice groups showed that LVEF in mice receiving either of the healthy CAC isolates was significantly higher than that in mice receiving 1 CAD CAC isolate or HBSS ($P<0.05$). The other CAD isolate did not achieve significant difference from 1 of the healthy CAC isolates but still differed from both of them by virtue of a lack of significant improvement in function over time. Similar results were obtained for end-systolic volume and end-diastolic volume, as shown in Figure 5B and 5C and Table 4.

## Transduction of Low-Function CAD CACs to Overexpress eNOS Improves Ex Vivo Function and Therapeutic Capacity

Because migration positively correlated with eNOS mRNA level and therapeutic potential, we attempted to augment

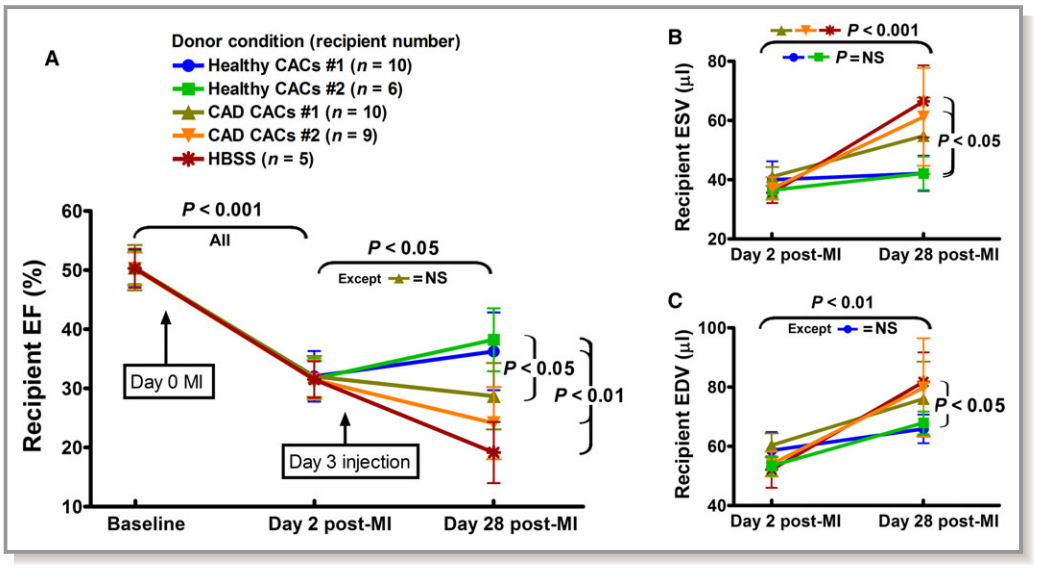


**Figure 5.** Strong correlation between circulating angiogenic cell (CAC) donor cardiovascular health, CAC ex vivo functional level, and therapeutic effect of CAC implantation into post–myocardial infarction (MI) mouse hearts. Graphs show (A) left ventricular ejection fraction (LVEF) (high number is better) and (B) end-systolic volume (ESV) and (C) end-diastolic volume (EDV) (low number is better) in 5 groups after injection with high-function or low-function CACs.

**Table 4.** Recipient Echocardiographic Parameters Before (Baseline and Day 2 After MI) and After (Day 28 After MI) the Implantation of Human CACs From Healthy Individuals and CAD Patients

| Donor Condition (Recipient Number) | EF, % | ESV, μL | EDV, μL |
|---|---|---|---|
| Healthy CACs #1 (n=10) | | | |
| Baseline | 50.23±2.96 | 34.44±4.06 | 69.11±6.13 |
| Day 2 post-MI | 31.99±4.25 | 40.05±6.20 | 58.67±6.22 |
| Day 28 post-MI | 36.19±6.55 | 42.16±5.96 | 65.95±4.85 |
| Healthy CACs #2 (n=6) | | | |
| Baseline | 50.28±2.76 | 32.59±4.35 | 65.68±8.81 |
| Day 2 post-MI | 31.60±3.44 | 36.54±2.59 | 53.45±3.25 |
| Day 28 post-MI | 38.15±5.31 | 42.19±5.60 | 67.98±3.83 |
| CAD CACs #1 (n=10) | | | |
| Baseline | 50.28±3.81 | 35.43±3.98 | 71.11±3.90 |
| Day 2 post-MI | 31.96±3.46 | 41.09±3.21 | 60.43±3.99 |
| Day 28 post-MI | 28.64±5.59 | 54.83±12.86 | 76.09±12.51 |
| CAD CACs #2 (n=9) | | | |
| Baseline | 50.11±2.75 | 32.45±5.26 | 64.84±8.27 |
| Day 2 post-MI | 31.40±3.29 | 37.12±3.81 | 54.07±4.22 |
| Day 28 post-MI | 24.09±6.08 | 61.21±16.47 | 79.84±16.62 |
| CAD CACs #2/Ad-eNOS (n=8) | | | |
| Baseline | 50.26±4.55 | 33.73±3.30 | 67.87±3.69 |
| Day 2 post-MI | 32.13±2.62 | 38.83±3.43 | 57.23±4.89 |
| Day 28 post-MI | 30.87±5.03 | 43.03±6.77 | 62.02±6.72 |
| Healthy CACs #2/Ad-eNOS-siRNA (n=9) | | | |
| Baseline | 50.24±4.55 | 34.52±4.75 | 69.23±5.45 |
| Day 2 post-MI | 31.66±1.23 | 39.20±3.63 | 57.38±5.32 |
| Day 28 post-MI | 26.70±6.13 | 52.84±14.09 | 71.34±13.45 |
| HBSS (n=5) | | | |
| Baseline | 50.18±3.29 | 34.59±4.42 | 69.30±5.73 |
| Day 2 post-MI | 31.45±3.07 | 35.67±3.48 | 52.15±6.10 |
| Day 28 post-MI | 19.15±5.15 | 66.40±12.11 | 81.76±9.98 |

The exact values are graphed in Figures 5 through 7. Errors are SD. See graphs in Figures 5 through 7 for statistical relationships. Ad-eNOS indicates adenoviral vector encoding endothelial nitric oxide synthase; CAC, circulating angiogenic cell; CAD, coronary artery disease; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; HBSS, Hanks buffered salt solution; MI, myocardial infarction; siRNA, small interfering RNA.

these qualities in the lowest-functional CAC isolate (CAD) by adenoviral transduction to overexpress eNOS, compared with a GFP virus control. In the least-functional CAC isolate ("CAD CACs #2" in Figure 5), eNOS was undetectable by immunoblotting, but after transduction with eNOS adenovirus, eNOS was clearly detectable at levels exceeding the natural level in HUVECs (Figure 6A), which we have reported to be higher than in CACs.[16] NO release from eNOS-transduced CAD CACs was elevated relative to that from untransduced and GFP-transduced CACs of the same isolate (0.24±0.01 versus 0.15±0.01 and 0.14±0.03 μmol/L, both $P<0.05$; Figure 6B). Migration was substantially elevated in the eNOS-transduced CAD CACs compared with nontransduced and GFP-transduced CACs (7.1±0.8 versus 4.2±0.4 and 4.6±0.4 cells/high-power field, both $P<0.05$; Figure 6C). When the CAD CACs were implanted intramyocardially into groups of SCID mice post MI, the therapeutic efficacy of the eNOS-transduced CAD CACs was improved relative to the untransduced CAD CACs and HBSS (Figure 6D, values in Table 4).

## eNOS Small Interfering RNA Knock-Down in High-Functional Young Healthy CACs Decreases Ex Vivo Function and Therapeutic Capacity

To mechanistically confirm the central requirement for eNOS in these functional and therapeutic properties, we asked conversely whether the most-functional CAC isolate (young healthy female) could be functionally and therapeutically impaired by eNOS knock-down via small interfering RNA (siRNA) adenoviral transduction. Transduction of HUVECs with this virus at 500 multiplicity of infection reduced eNOS protein level to 22% of untransduced levels as determined by quantitative immunoblotting (Figure 7A). When healthy CACs ("Healthy CACs #2" in Figure 5) were transduced with the siRNA virus, NO release was markedly decreased compared with untransduced and GFP virus–transduced CACs of the same isolate (0.07±0.02 versus 0.39±0.04 and 0.36±0.06 μmol/L respectively, both $P<0.05$; Figure 7B). Migration was substantially reduced in the siRNA-transduced CACs compared with the untransduced and GFP-transduced CACs (6.3±1.3 versus 19.6±2.9 and 17.6±2.5 cells/high-power field, respectively, both $P<0.01$; Figure 7C). When these healthy donor CACs were implanted into post-MI mouse hearts, the therapeutic efficacy of the siRNA-transduced CACs was impaired relative to the untransduced group but not to the level of the HBSS-injected control group (Figure 7D, values in Table 4).

## Lack of Long-Term Persistence of CACs in the Myocardium Despite Functional Improvement

To confirm ability to detect human CACs in mouse heart tissue, a pilot experiment was performed in which CACs were preloaded with cytoplasmic Cell Tracker green dye before implantation into SCID mouse hearts. Immunofluorescence staining of tissue sections for HLA detected cells with cytoplasmic cell tracker and surface HLA staining (Figure 8). However, despite clear functional benefits of CAC implantation even 25 days postimplantation (28 days post MI), we did not detect CACs persisting in the tissue at that late time point.

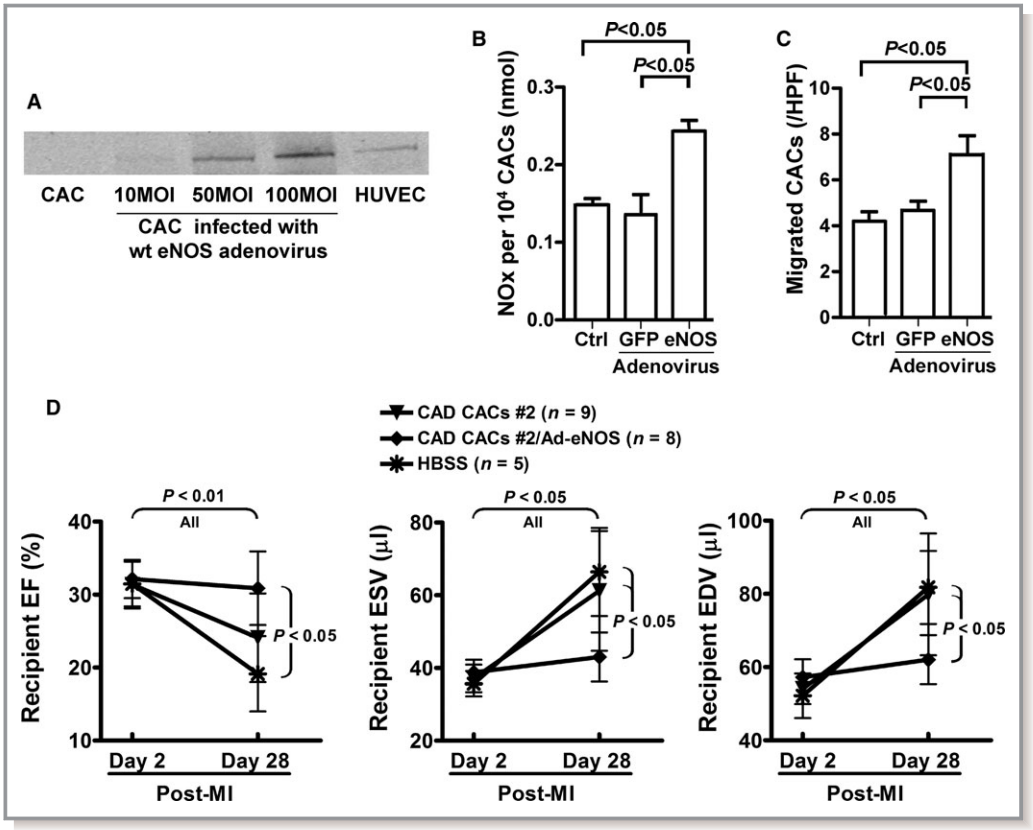


**Figure 6.** Overexpression of endothelial nitric oxide synthase (eNOS) in coronary artery disease (CAD) circulating angiogenic cells (CACs) improves functional and therapeutic efficacy. A, CAD CACs transduced with eNOS adenovirus overexpress eNOS protein detected by Western blot, (B) produce more nitric oxide (NO), and (C) migrate more in culture. D, These functional improvements translate into better therapeutic efficacy in the post–myocardial infarction (MI) heart.

## Significant Correlation was not Observed Between CAC Condition, Blood Vessel Density, and Infarct Size at 25 Days Postimplantation

Histological analysis of tissue sections at the infarct border zone of day 28 post-MI hearts from different representative CAC implantation groups revealed no correlation with density of capillaries and arterioles (Figure 9). Expected relationships were observed for infarct scar size based on the cardiac functional data; however, the results did not approach significance because of the underpowered group size and the loss of several frozen negative control hearts (Figure 10; see Discussion).

## Discussion

The finding that CACs from different donors possess a range of therapeutic potential underscores the challenges of autologous cell therapy, in which the therapeutic agent is different for each patient.[1,26,27] Our results indicate that age and potentially CAD can influence therapeutic properties of CACs for the treatment of MI, because of multiple levels of eNOS regulation that lead to differential NO release, and that functional capacity of individual patient CAC isolates in culture predicts their therapeutic efficacy. Correlation of ex vivo function and therapeutic capacity has been a common theme in autologous CAC studies,[21] supporting the importance of enhancing poorly functional cells. Not only could we make therapeutically impaired CACs from old CAD patients more therapeutic by eNOS gene therapy, but also highly therapeutic CACs from young healthy people were made less therapeutic by reducing eNOS transcription, confirming cause and effect.

It is notable that while migration toward VEGF is a well-established assay of CAC functional properties in culture,[6–9,21] the VEGF itself may be superfluous; as we report here, correlation of donor characteristics with migration toward VEGF is driven by the intrinsic migratory capacity of the cells that is apparent even in the absence of VEGF. Isolation of VEGF-directed chemotactic ability, achieved by normalizing migration toward VEGF to migration without VEGF, abolishes the correlation. Our observation of this effect in CAD-driven migratory impairment is consistent

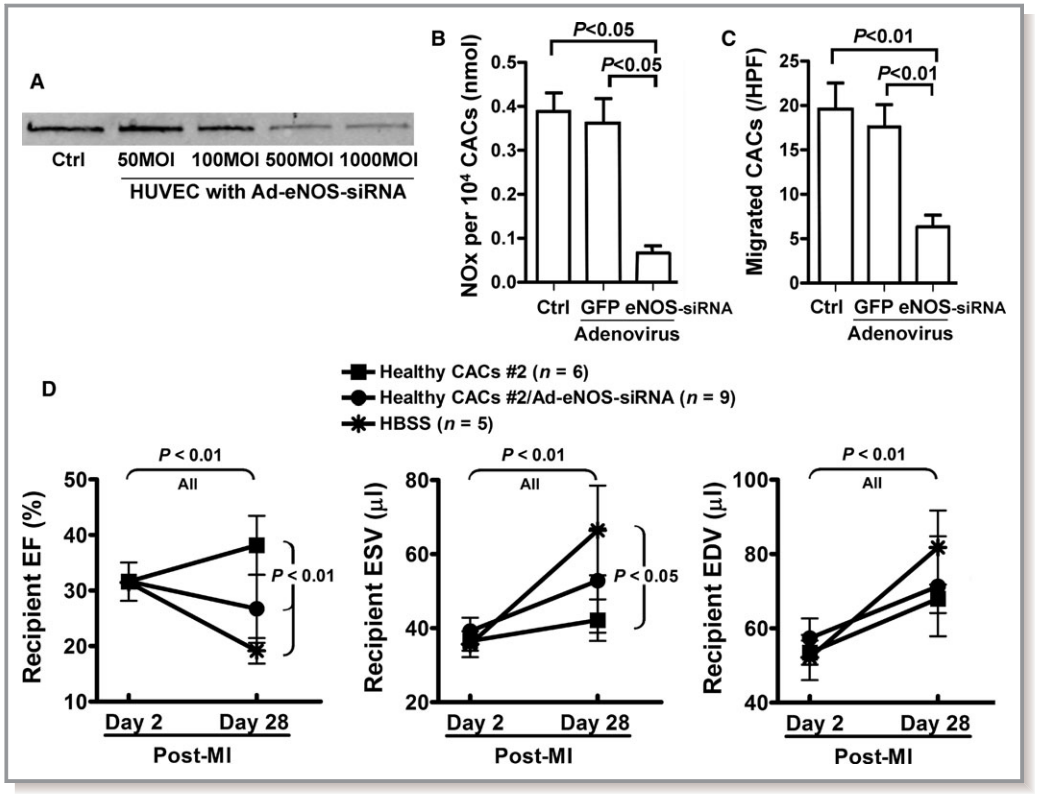


**Figure 7.** Endothelial nitric oxide synthase (eNOS) small interfering RNA (siRNA) knock-down in highly functional circulating angiogenic cells (CACs) reduced functional and therapeutic efficacy. A, Immunoblot of human umbilical vascular endothelial cells (HUVECs) transduced with eNOS siRNA adenovirus confirm that this siRNA virus reduces the expression of eNOS protein. B and C, High-function young healthy female CACs transduced with the eNOS siRNA virus produce less NO and migrate less in culture. D, These functional impairments translate into worse therapeutic efficacy in the post–myocardial infarction (MI) heart.

with our previous observations in a smaller pilot CAD group[16] and in the healthy subpopulation from the current cohort in which we observed a correlation between reduced migratory capacity and higher fasting plasma glucose levels in the healthy (nondiabetic) donor group, which we have published separately.[47]

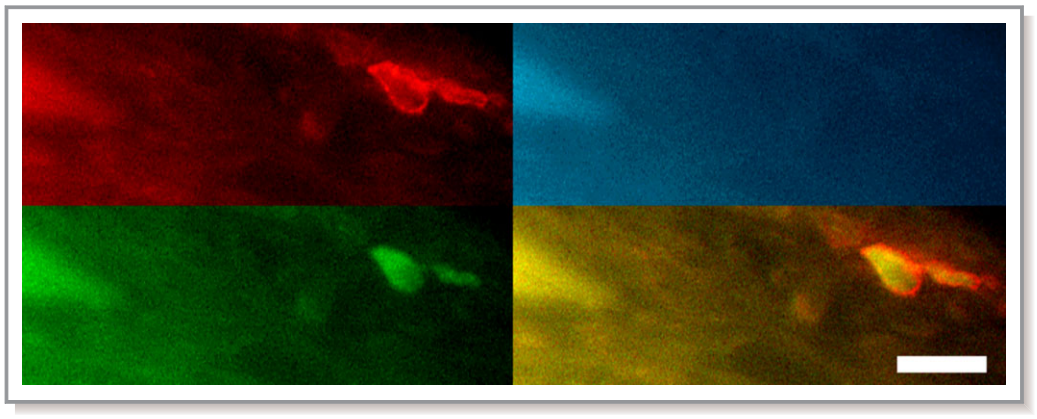

**Figure 8.** Detection of circulating angiogenic cells (CACs) by human-specific immunofluorescence 3 days after implantation. Human CACs at day 3 postimplantation into myocardium in a pilot experiment are identified by immunofluorescence staining of tissue sections for the human surface marker HLA (red) and cytoplasmic cell tracker (green). Red=HLA, green=cell tracker dye, blue=open channel for autofluorescence, last panel=red/green merged. Bar=20 μm.

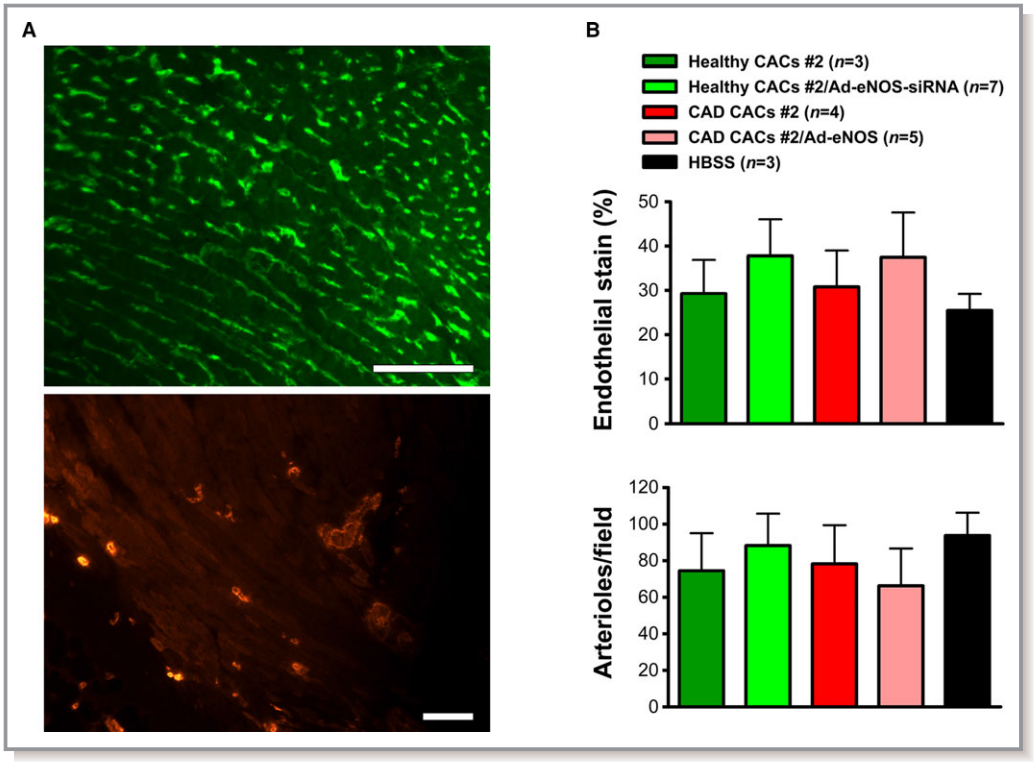


**Figure 9.** Blood vessel density in border zone myocardium at 28 days post myocardial infarction (MI). A, Representative photomicrographs showing endothelium stained with fluorescent isolectin B4 (green pseudo-color) and α–smooth muscle actin immunostaining (red). Bars=100 μm. B, Capillary density was approximated by measuring percentage of ×20 objective microscope fields that were positive for stain. Arteriole density including venules was expressed as number of smooth muscle actin–positive vessels per ×10 objective power microscope field. All counts were corrected for gaps in the tissue. No significant differences were observed.

Ward et al[24] published a relevant report that also examined functional and therapeutic properties of CACs from CAD patients. Our studies agree on several points, although they have different goals and reveal different findings, which we compare and contrast as follows. Ward et al examined high– and low–cardiovascular risk donors but did not distinguish

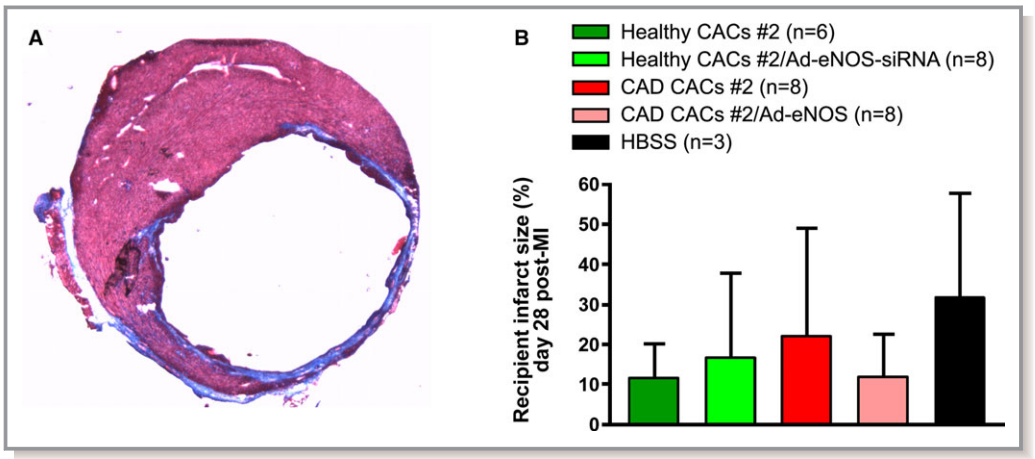


**Figure 10.** Histological measurement of fibrotic zone and calculation of infarct scar size. A, Representative staining of infarct scar with Masson trichrome. B, Quantitation of infarct size defined as percent length of fibrotic scar within the circumference of the left ventricle (LV), averaged over the length of the LV. Significance of differences was not achieved as a result of the underpowered group size, as the number of circulating angiogenic cells (CACs) were limiting for each group, but the trend was consistent with our functional results.

between the 2 different levels of eNOS regulation that we report to be differentially influenced in different populations. They used migration toward VEGF as a diagnostic test for CAC function, while our results extend this concept and suggest that functionality actually depends on intrinsic migratory capacity rather than chemotactic response to VEGF that is traditionally presumed to be the limiting functional property. The Ward et al study evaluated therapeutic potential of CAD CACs transduced to express eNOS in the classic mouse hindlimb ischemia model, whereas we have explored the effects of age, disease, and engineered modulation of eNOS expression on the therapeutic potential for treatment of MI. Notably, we not only have demonstrated improvement of poorly functional CACs by eNOS transduction but also have shown reciprocally that highly functional CACs are impaired by eNOS knock-down. Of note, we previously reported that inducible NOS mRNA is not detectable in CACs from healthy subjects or CAD patients.[16] These findings taken together confirm the key role played by eNOS in the ability of CACs to influence post-MI left ventricular function.

Jakob et al[48] reported that microRNAs miR-126 and -130a are similarly reduced in CACs from heart failure patients and that manipulation of miR-126 expression influenced CAC therapeutic efficacy in a mouse MI model. This finding suggests that multiple molecular deficiencies may be targeted singly or in concert to improve poorly functional CACs.

It is important to define our CACs in the context of previous studies of similar cell populations. Based on isolation and expansion conditions and on phenotypic characterizations, the CACs that we and others isolate appear to be the same population as those studied under the EPC designation by Vasa et al[8] in their seminal report relating ex vivo function to cardiovascular risk, the "circulating progenitor cells" in a clinical study from the same group,[22] the "angiogenic early outgrowth cells" studied by Jakob et al,[48] and the EPCs studied by Kawamoto et al.[19] However, they are not the same population as that studied by the latter group subsequently, which was selected for CD31 or CD34 expression.[49] CACs are distinct from comparably cultured bone marrow mononuclear cells, for which eNOS overexpression did not enhance the ability to improve post-MI cardiac function,[50] and from mobilized $CD34^+$ bone marrow cells, which are the subject of a phase 3 clinical trial for angina.[51] The population-level phenotypic shift that we observed in impaired CAD CACs toward monocytic gene expression and modestly away from endothelial gene expression (Figure 4) may be relevant to recent follow-up studies of the Timing in Myocardial Infarction Evaluation (TIME) clinical trial of bone marrow cell therapy for MI,[52] in which better clinical outcomes of MI were correlated with increased CD31 expression and decreased CD11b expression on the bone marrow cells.[53,54]

A limitation of our study is that we were unable to completely age-match the older healthy subjects to the CAD patients, the latter of which had a higher mean age (71.5±2.6 years) than the older healthy group (51.1±1.5 years). Therefore, some of what appear as CAD-driven effects may actually be driven by older age. However, this would not negate the finding that eNOS transcription was driven by age rather than CAD, and our suggestion that the other functional impairments were driven primarily by CAD is consistent with previous reports of correlations between CAC function and cardiovascular risk factors.[8,12] This interpretation is further supported by our observation that within the CAD group, there was no correlation between age and eNOS transcription levels. Moreover, comparisons of the younger groups of a single sex to the older, mixed-sex CAD group are limited in their ability to isolate key factors.

Another limitation is that because our cardiac functional assays relied on day-28 end points and because patient CACs were of limited number, histological assessment of vessel density was performed only at that point, which may have been too late to detect vascular differences before vessel density normalized in the fully remodeled tissue, as we observed previously in the case of implanted bone marrow cells.[44] The absence of detectable implanted CACs at day 28 is also reminiscent of that study, in which we observed rapid loss of implanted cells over the first several days. We intentionally did not harvest hearts at earlier times for assessment of cell number because patient isolates were limited in quantity, and we gave higher priority to ensuring statistically sufficient end point group size for functional analysis; thus, we were not able to adequately evaluate the effect on early angiogenic response in this already complex study. This is an important point, because our lack of observation of increased angiogenesis after 25 days (28 days post MI) from these "circulating angiogenic cells" does not indicate that angiogenesis was not an important therapeutic mechanism during the earlier crucial phase of cardiomyocyte apoptosis and myocardial remodeling. Alternatively, they may have played an immunomodulatory role. A study of the earlier events after CAC implantation into myocardium will be informative, but was infeasible to include in this study.

The limiting number of CACs from CAD patients referred to earlier suggests a potential additional challenge to autologous CAC therapy—the ability to harvest enough cells for treatment. This may present problems to ultimate clinical translatability, especially if a large number of cells are required for intravenous infusion. Localized injection into tissues obviates this problem somewhat. Still, it is important to note that cell number was more limiting for our experiments than for clinical translation for 2 reasons. First, the amount of blood that is acceptable to remove from a diseased research study participant is smaller than that for a patient who stands to benefit from the therapy.

Second, the cells that we isolated from each participant needed to be used for multiple experimental conditions that included ex vivo functional tests, transduction of multiple subpopulations from a given isolate, and reexpansion of each population. Still, as with all cell therapy preclinical models, ultimate success will hinge on the ability to scale up for human use. Furthermore, any cell therapy strategy that involves many days of expansion in culture will not be feasible for treatment of an acute condition, so clinical success would depend as well on appropriate balance between number of cells required and length of time post MI during which they could still influence outcomes.

While our group size was powered sufficiently to detect differences in cardiac function, it was not large enough to enable sufficient power to detect significant differences in fibrotic infarct scar size. This limitation was exacerbated by large standard deviations. Notably, the mean infarct sizes from each group displayed precisely the relationship that would be expected based on our functional results even though statistical significance was not achieved (Figure 10). That is, all CAC groups displayed infarct size smaller than the negative control group, the healthy CAC group displayed smaller infarct size than the CAD CAC group, siRNA knock-down of eNOS resulted in larger infarct size, and transduction with eNOS virus resulted in smaller infarct size. Because of the lack of power and significance, we cannot conclude from this experiment that infarct size was influenced by the condition of the CACs. Nonetheless, the trend was consistent mechanistically with the preservation of at-risk border zone myocardium that resulted from similar implantation of bone marrow cells in our earlier study[44] and is congruent with the tendency of infarct size to inversely correlate with LVEF as we have previously observed[45] ($r=-0.85$, $P<0.01$ for infarct size and LVEF in that study).

To summarize, we have shown that CACs from older and CAD donor groups release less NO than those from young healthy donors, because of distinct reductions in both eNOS mRNA levels and eNOS protein levels. This is associated not only with reduced intrinsic migration (rather than specific deficits in VEGF response) but also with reduced therapeutic capacity when injected into post-MI mouse hearts. All of these negative effects in CAD CACs can be improved by ex vivo eNOS gene therapy and, conversely, the functional and therapeutic effects of CACs from young healthy donors can be impaired by eNOS siRNA transduction. These findings underscore the central role played by eNOS in CAC function and in the therapeutic potential of eNOS-engineered patient CACs for autologous treatment of cardiovascular disease.

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## Disclosures

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# Overexpression of Nitric Oxide Synthase Restores Circulating Angiogenic Cell Function in Patients With Coronary Artery Disease: Implications for Autologous Cell Therapy for Myocardial Infarction

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