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TRANSLATIONAL AND CLINICAL RESEARCH

Restoring Akt1 Activity in Outgrowth Endothelial Cells From South Asian Men Rescues Vascular Reparative Potential

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ABSTRACT

Recent data suggest reduced indices of vascular repair in South Asian men, a group at increased risk of cardiovascular events. Outgrowth endothelial cells (OEC) represent an attractive tool to study vascular repair in humans and may offer potential in cell-based repair therapies. We aimed to define and manipulate potential mechanisms of impaired vascular repair in South Asian (SA) men. In vitro and in vivo assays of vascular repair and angiogenesis were performed using OEC derived from SA men and matched European controls, prior defining potentially causal molecular mechanisms. SA OEC exhibited impaired colony formation, migration, and in vitro angiogenesis, associated with decreased expression of the proangiogenic molecules Akt1 and endothelial nitric oxide synthase (eNOS). Transfusion of European OEC into immunodeficient mice after wire-induced femoral artery injury augmented re-endothelialization, in contrast with SA OEC and vehicle; SA OEC also failed to promote angiogenesis after induction of hind limb ischemia. Expression of constitutively active Akt1 (E17KAkt), but not green fluorescent protein control, in SA OEC increased in vitro angiogenesis, which was abrogated by a NOS antagonist. Moreover, E17KAkt expressing SA OEC promoted re-endothelialization of wire-injured femoral arteries, and perfusion recovery of ischemic limbs, to a magnitude comparable with nonmanipulated European OEC. Silencing Akt1 in European OEC recapitulated the functional deficits noted in SA OEC. Reduced signaling via the Akt/eNOS axis is causally linked with impaired OEC-mediated vascular repair in South Asian men. These data prove the principle of rescuing marked reparative dysfunction in OEC derived from these men. STEM CELLS *2014;32:2714–2723*

INTRODUCTION

Outgrowth endothelial cells (OEC; also known as late outgrowth endothelial progenitor cells or endothelial colony forming cells) possess the functional properties of vascular endothelium, while exhibiting a progenitor hierarchy and the capacity for significant ex vivo expansion [1]. These properties, and the potential to easily derive autologous cells, make them an ideal tool to study indices of endothelial repair in samples from apparently healthy individuals at risk of cardiovascular events; moreover, they possess potential in translation as a cardiovascular repair therapy. A growing body of evidence supports reduced vascular repair in association with diabetes and insulin resistance, disorders linked with persistently poor cardiovascular mortality and morbidity [2]. Many other studies have shown vascular dysfunction and insulin resistance from childhood onwards in people of South Asian ethnicity, and even in early adulthood, this group is at increased relative risk of major vascular events [3–5]. Moreover, in later life, South Asian ethnicity is associated with more diffuse coronary artery disease and retinal microvascular rarefaction [6, 7]. Our previous work has demonstrated impaired vascular function and a reduction in basal and exercise mobilized circulating progenitor cells in healthy young South Asian men as a result of reduced nitric oxide (NO) bioavailability [8, 9]. Importantly, one of the subsets of circulating progenitor cells, we noted to be reduced in South Asian men (CD34+/CD45−) is acknowledged as the

likely primitive source of OEC [10, 11] We set out to define the reparative capacity of OEC derived from South Asian men, versus a control cohort of white European men, and then establish how OEC dysfunction might be reversed.

Materials and Methods

Subject Recruitment

Healthy white European and South Asian men were recruited by poster advertisement. Ethnicity was defined by subjects from a list used in UK health-care monitoring [12]; subjects defining their ethnicity within subdivisions of "Asian or British Asian" were defined as South Asian; those within the subdivisions of "white" were defined as white European. Subjects were eligible for inclusion if aged 18–40 years, free from any chronic illness, not taking prescription medication, nonsmokers within the past year, and free from hypertension (blood pressure $>$ 160/90 mmHg), diabetes, and hypercholesterolemia (total cholesterol $>$ 7 mmol/L). The homeostasis index of insulin resistance was calculated as published previously [8]. A total of 12 white European and 12 South Asian men were included. All participants provided written informed consent, according to the declaration of Helsinki; ethical approval was provided by the Harrogate and Leeds (Central) research ethics committees.

OEC Culture

Peripheral blood mononuclear cells were harvested from 35 ml venous blood using density gradient centrifugation (Ficoll, GE Healthcare, UK; www.gelifesciences.com), and suspended in EGM-2 medium with EGM-2 Bullet kit (Lonza, UK; http://www.lonza.com) and 10% fetal calf serum (FCS). Cells were seeded on fibronectin and medium replaced daily for the first week, then on alternate days. After 3–4 weeks, cells with cobblestone morphology developed in OEC colonies [13]; cells exhibited contact inhibition and were capable of serial passage. Endothelial phenotype was confirmed using flow cytometry, demonstrating almost universal expression of the endothelial markers CD31, CD144, CD146, and CD309, along with almost absent expression of the pan-leukocyte marker CD45 and the monocyte marker CD14 (Supporting Information Fig. S1).

Flow Cytometry

OEC were washed twice in phosphate-buffered saline (PBS) and then incubated with FcR blocker (Miltenyi Biotech, UK; https://www.miltenyibiotec.com). Appropriate concentrations of the following antibodies were then applied in separate tubes: CD31-FITC, CD146-FITC, CD309-PE, CD45-APC, CD14-APC (Miltenyi Biotech, UK), or CD144-PE (BD Biosciences, UK; www.bdbiosciences.com); class and isotype specific control fluorescent antibodies were used to gate nonspecific fluorescence. Cells were analyzed using a BD LSR-Fortessa cytometer with FACS DiVa6 software (BD Biosciences, UK) and histogram overlays were formatted using FlowJo v.7 (Ashland, OR; www.flowjo.com).

Proliferation

Viable OEC were enumerated using hemocytometry at passage 2 and 4 (P2 and P4), with exclusion of Trypan blue stained nonviable cells. The number of population doublings between these points was calculated according to the equation $\log_2$ (population at P4/population at P2); the time in days between P2 and P4 was divided by this figure to derive a population doubling time.

Migration and Invasion Assays

An amount of 4×10^4 OEC suspended in basal EGM-2 medium (1% FCS, without other additives) were placed in the upper compartment of modified Boyden chamber apparatus. The lower compartment contained EGM-2 with 50 ng/ml vascular endothelial growth factor ($VEGF_{165}$; R&D Systems, UK; www.rndsystems.com), 50 ng/ml insulin-like growth factor-1 (IGF-1; R&D Systems, UK), or vehicle alone (control). Wells, in triplicate, were incubated for 24 hours. Membranes were fixed in 70% ethanol before mechanical removal of cells on the upper surface, and hematoxylin/eosin staining; migrant OEC were enumerated in 10 high power fields (HPF: 1.52 mm^2) and expressed as the mean value per HPF. Invasion assays were carried out in an identical manner, other than using Boyden chamber inserts precoated with Matrigel (Corning, UK; www.corning.com), to define VEGF-directed invasion, versus vehicle control.

In Vitro Angiogenesis

OEC (5×10^4 cells per milliliter in EGM-2 with 50 ng/ml $VEGF_{165}$ and 1% FCS) were seeded in triplicate on Matrigel-coated plates for 24 hours; in a subset of experiments 0.1 mmol/L L-N^G-nitro-L-arginine methyl ester (L-NMMA), a nitric oxide synthase (NOS) antagonist, was also added to culture medium. Tubule formation was defined as participation in contiguous polygonal structures; mean number of tubular structures per HPF was calculated based upon data collected in 10 HPF.

Senescence

Sub-confluent third passage LEPC were studied using a senescence associated β-galactosidase staining kit (Cell Signaling, UK; www.cellsignal.com); senescent EPCs were counted by two-blinded independent operators (inter-observer agreement: $r = 0.99$) using Image J software (National Institutes of Health, MD; imagej.nih.gov/ij/) and expressed as percentage total cells.

Survival Assay

Sub-confluent OEC were placed in basal EGM-2 medium with 0.5% heat inactivated FCS, 20 ng/ml recombinant human tumor necrosis factor (TNF)-α (R&D systems, UK), and 500 μM hydrogen peroxide (Sigma), then incubated for 24 hours at 37°C in 1% O_2 and 5% CO_2, to mimic an ischemic tissue milieu [14]. LDH activity was measured in the conditioned medium and normalized to total cellular LDH using a commercially available kit (Sigma, UK; www.sigmaaldrich.com).

Western Blotting

Total protein of 30 mcg from cellular lysates was loaded onto 4–12% SDS-PAGE gels (Invitrogen, UK; www.lifetechnologies.com) and electrophoresed under reducing conditions before transfer to PVDF membranes. Membranes were probed with primary antibody (anti-Akt [Cell Signaling Technology; Boston, MA], anti-endothelial NOS (eNOS) [BD Biosciences, UK], anti-phospho S473 Akt [Cell Signaling Technology; Boston, MA],

anti-phospho S1177 eNOS [BD Biosciences, UK], or anti-beta-actin [Santa Cruz Biotechnology, Dallas, TX; www.scbt.com]) overnight in 5% skimmed milk in tris buffered saline (TBS)-Tween buffer, followed by horse radish peroxidase (HRP)-conjugated secondary antibody for an hour. Proteins were visualized using enhanced ECL kit (Amersham Biosciences, UK; www.gelifesciences.com).

Polymerase Chain Reaction

Total RNA was extracted from OEC using TRIZOL (Sigma-Aldrich, UK). Equal quantities of RNA were reverse transcribed using reverse transcription kit (Applied Biosystems, UK; www.lifetechnologies.com) following manufacturer's protocol. Real-time PCR was performed using the following primers using SYBR-based assay (Applied Biosystems, UK: 7900HT) and beta-actin normalization:

eNOS forward, 5′-CTG-GAG-CAC-CCC-ACG-CT-3′
eNOS reverse, 5′-AGC-GGT-GAG-GGT-CAC-ACA-G-3′
Akt1 forward, 5′-CCT-TCC-TCA-CAG-CCC-TGA-AGT-3′
Akt1 reverse, 5′-CCG-GGA-CAG-GTG-GAA-GAA-C-3′
Beta-actin forward, 5′-CGT-GAA-AAG-ATG-ACC-CAG-ATC-A-3′
Beta-actin reverse, 5′-TGG-TAC-GAC-CAG-AGG-CAT-ACA-G-3′

Akt Activity

Akt kinase activity was analyzed by nonradioactive immunoprecipitation-kinase assay according to manufacturer's protocol (Cell Signaling Technology; Boston, MA). Cell extracts of 30 mcg were incubated with immobilized Akt 1G1 monoclonal antibody. After extensive washing, the kinase reaction was performed at 30°C for 30 minutes in the presence of 200 μM cold ATP and GSK-3 substrate. Phospho-GSK-3 was measured by Western blot, using phospho-GSK-3α/β (Ser-21/9) antibody.

Animals

A 9- to 13-week-old (weight 25–33 g) male immunodeficient CD1 nude mice (Charles River Labs, UK; www.criver.com), housed in isolators with 12-hour light–dark cycle, were used in all experiments; standard diet was provided *ad libitum*. Experiments were performed under license from the UK Home Office, observing standard animal welfare regulations.

Vascular Injury and Analysis

As published [15], mice were anesthetized with isoflurane before femoral arteriotomy and three 1.5-cm passages of a 0.014-inch-diameter angioplasty guidewire (Hi-Torque Cross-IT 200XT; Abbott Vascular, UK; www.abbottvascular.com); the vessel was then ligated and skin closed. The contralateral artery underwent sham operation, without arteriotomy or wire injury. Animals received buprenorphine 0.25 mg/kg. $3x10^5$ CMDiI (Invitrogen, UK) labeled OEC in EBM2 (or EBM2 without cells, as control) were transfused into the external iliac vein immediately after injury. Injured and contralateral femoral arteries were explanted 4 days later after injection of 50 μl 5% Evans Blue dye via the inferior vena cava and perfusion fixation with 4% paraformaldehyde in PBS. Re-endothelialization was assessed in en face specimens, defined as the region of absent Evan's blue staining in relation to a total area of a 5-mm section of vessel, commencing 5 mm distal to the aortic bifurcation. Vessels were then blocked (Serum free protein block, DAKO, UK; www.dako.com) and incubated with a rabbit polyclonal antibody to mouse/human CD31 (ab28364, Abcam, UK; www.abcam.com) followed by a goat polyclonal anti-rabbit conjugated to Chromeo642 (ab60319, Abcam, UK), then mounted on slides with DAPI (DAPI-Fluoromount-G, Southern Biotech, AL; www.southernbiotech.com) to define nuclei. Confocal microscopy (Zeiss LSM 510 META Axioplan 2 Zeiss, UK; www.zeiss.co.uk) was used to count adherent OEC, defined by nuclei with peripheral DiI and Chromeo642 fluorescence, per square millimeter (mm^2). In a separate series of experiments, identical vascular injury and transfusion protocols were performed, and vessels were explanted 4 weeks later, with subsequent confocal analysis to determine long-term engraftment of OEC. All murine studies and analysis were performed by researchers blinded to "treatment" allocation.

Hind-Limb Ischemia and Analysis

Mice were anesthetized with isoflurane before dissecting the left femoral vessels, ligating the femoral artery proximally at the inguinal ligament and distally at the bifurcation to saphenous and popliteal vessels, and excising the intervening arterial segment. Six hours later, [16] 3×10^5 CMDiI (Invitrogen, UK) labeled OEC in EBM2 (or EBM2 without cells, as control) were transfused into the right femoral vein at the time of contralateral sham surgery. Laser Doppler analysis (Moor LDI2-HR, Moor systems, UK; gb.moor.co.uk) of ischemic and sham-injured limbs was then performed, in a temperature controlled environment, to confirm induction of ischemia. Images were analyzed (MoorLDI software, Version 5.3, Moor systems, UK) to derive an ischemic to non-ischemic limb perfusion ratio, based upon flux below the level of the inguinal ligament. On day 14, laser Doppler analysis was repeated to define limb perfusion recovery, before perfusion fixation with 4% paraformaldehyde and harvesting of ischemic and contralateral gastrocnemius muscle. Muscle specimens were embedded in OCT (Tissue-Tek OCT compound, Sakura, Netherlands) before snap freezing in liquid nitrogen and cryosectioning at 10 μm thickness. Specimens were then blocked (Serum free protein block, DAKO, UK) and incubated with a rabbit polyclonal antibody to mouse/human CD31 (ab28364, Abcam, UK) followed by a goat polyclonal anti-rabbit conjugated to Chromeo642 (ab60319, Abcam, UK), then mounted on slides with DAPI (DAPI-Fluoromount-G, Southern Biotech, AL) to define nuclei. Confocal microscopy (Zeiss LSM 510 META Axioplan 2) was used to count engrafted OEC, defined by nuclei with peripheral DiI and Chromeo642 fluorescence, per mm^2.

SIN-Lentiviral Vector Production and Transduction

pSINCSGWdlNot1 was a kind gift of Dr. Yashiro Idea (Mayo Clinic, Minnesota) [17]. The enhanced green fluorescent protein (EGFP) cassette of pSINCSGWdlNot1 was replaced by E17KAkt to generate pHVSLS2. The SIN-lentiviral vectors were generated in accordance to established protocols [18], and were titred using QuickTiter Lentivirus Titer Kit (Lentivirus-Associated HIV p24 ELISA kit [Cell Biolabs; San Diego, CA; www.cellbiolabs.com]) in accordance with manufacturer's protocol. Semi-confluent second passage OEC were transduced at one moi (multiplicity of infection) of appropriate lentiviral vector for use 4 days later.

Akt1 Silencing

Sub-confluent OEC were transfected with 20 nm Akt1 or control scrambled siRNA (Invitrogen) using Lipofectamine

RNAiMax (Invitrogen), according to the manufacturer's instructions. Confirmation of Akt1 knockdown and functional studies, were conducted 48 hours later.

Statistical Analysis

Data are presented as mean (standard error of mean) and groups compared with Student's *t*-tests or paired *t*-tests as appropriate. Statistical significance is defined as $p < .05$.

Results

We recruited a cohort of apparently healthy SA men and matched white European (WE) controls ($n = 12$ per group) to assess basal abundance and function of OEC, before assessing strategies to optimize their therapeutic efficacy. Groups were well matched for demographic factors and established cardiovascular risk factors, though relative insulin resistance was apparent in the SA cohort (Table 1).

Table 1. Demographic and cardiovascular risk data

	South Asian	White European	*p* value
Age (years)	30.9 (1.2)	29.7 (1.1)	NS
Body mass index (kg/m^2)	24.1 (0.7)	22.4 (0.5)	NS
Waist–hip ratio	0.85 (0.01)	0.84 (0.01)	NS
Systolic blood pressure (mmHg)	116 (2.3)	116 (2.5)	NS
Diastolic blood pressure (mmHg)	70 (1.6)	69 (1.6)	NS
Total cholesterol (mmol/l)	4.6 (0.1)	4.7 (0.1)	NS
Triglycerides (mmol/l)	1.1 (0.1)	1.2 (0.1)	NS
Glucose (mmol/l)	4.8 (0.1)	4.6 (0.1)	NS
Insulin (mu/l)	5.6 (0.8)	2.9 (0.3)	.009
HOMA-IR	1.2 (0.2)	0.6 (0.1)	.008

All data are expressed as mean (SEM).
Abbreviations: HOMA-IR, homeostasis index of insulin resistance; NS, nonsignificant.

SA OEC are Dysfunctional In Vitro

OEC colony formation was markedly reduced in SA men (Fig. 1A; Supporting Information Fig. S1A), suggesting a lower abundance of circulating progenitors; OEC phenotype was confirmed using flow cytometry, showing almost universal expression of endothelial markers CD31, CD144, CD146, and CD309, along with almost absent expression of the pan-leukocyte marker CD45 and monocyte marker CD14 (Supporting Information Fig. S1C). The surface marker profile of OEC derived from WE and SA men was comparable (Supporting Information Fig. S1D). The proliferative rate and migration toward physiologically relevant stimuli (VEGF and IGF-1) were blunted in SA OEC (Fig. 1B, 1C; Supporting Information Fig. S2). VEGF stimulated in vitro angiogenesis, measured by tubule formation on Matrigel, was also significantly reduced (Fig. 1D; Supporting Information Fig. S2). SA OEC were also more senescent as a population as defined by β-galactosidase staining (Fig. 1E; Supporting Information Fig. S2). The capacity of OEC derived from WE and SA men to invade a Matrigel layer was similar (Fig. 1F). Concentrations of the proangiogenic molecules protein kinase-B (Akt1) and eNOS were markedly reduced in SA OEC at protein level (including the "activated" S473 and S1177 phosphorylated forms, respectively) (Fig. 2A–2D). *eNOS*, but not *Akt1*, mRNA was less abundant in SA OEC (Supporting Information Fig. S3).

SA OEC Are Dysfunctional In Vivo

To probe the in vivo relevance of our in vitro studies, equal numbers of OEC were transfused intravenously into CD1 immunodeficient mice immediately after wire-induced luminal injury of the common femoral artery, with vehicle medium serving as control. After 4 days, re-endothelialization was assessed using Evans blue staining of the injured and contralateral noninjured vessel to indicate persistent endothelial

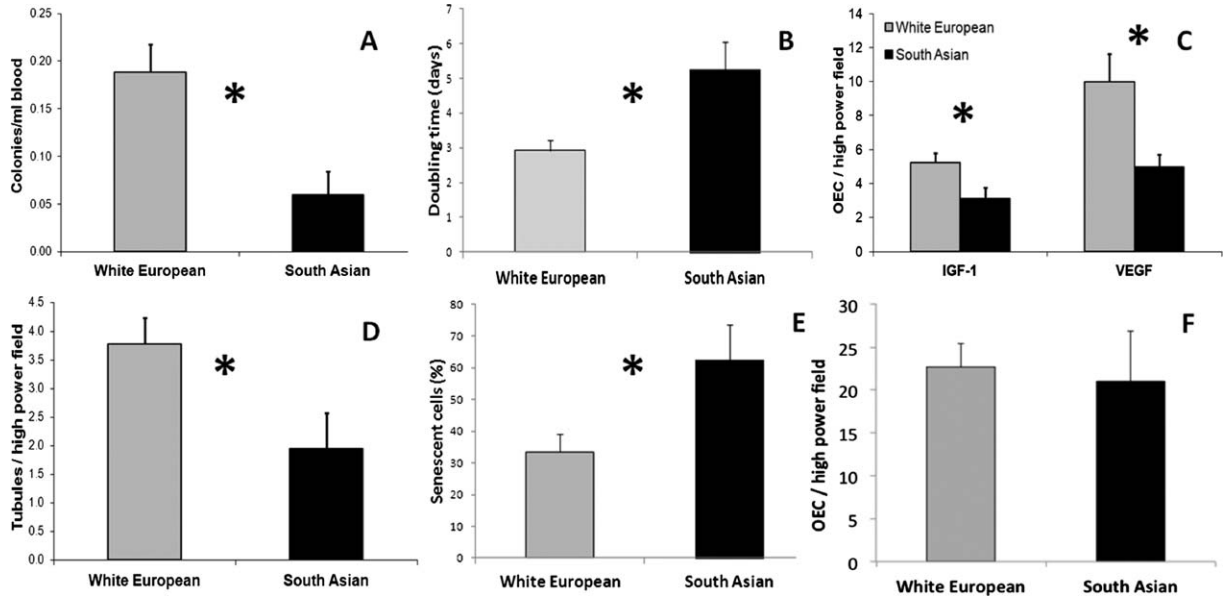

Figure 1. In vitro characterization of outgrowth endothelial cell (OEC) abundance and function. In South Asian men, compared with white European controls, OEC. **(A):** colony formation is reduced ($n = 8$); **(B)** population doubling time is prolonged ($n = 8$); **(C)** migration to vascular endothelial growth factor and insulin-like growth factor-1 is impaired ($n = 8$); **(D)** in vitro angiogenesis is reduced ($n = 8$); **(E)** SENESCENCE is increased ($n = 8$); **(F)** invasion through a Matrigel layer is comparable. All data are displayed as mean ± SEM; *, $p < .05$. Abbreviation: OEC, outgrowth endothelial cell.

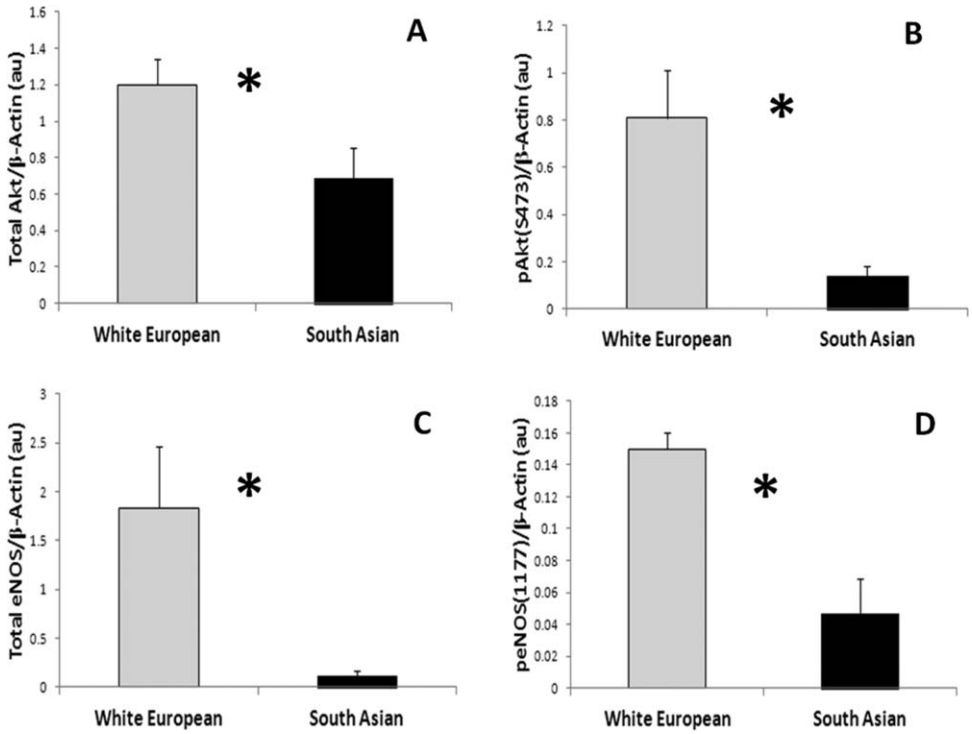

Figure 2. Basal- and insulin-stimulated phosphorylation of Akt and endothelial nitric oxide synthase (eNOS). **(A, B):** Total and phospho-S473 Akt are reduced ($n = 7$); **(C, D)** total and phospho-S1177 eNOS are reduced ($n = 7$). All data are displayed as mean ± SEM; *, $p < .05$.

denudation (Fig. 3A). OEC derived from WE subjects significantly augmented re-endothelialization, whereas the femoral arteries of mice exposed to SA OEC were indistinguishable from those receiving vehicle (Fig. 3B). Confocal microscopy revealed fewer adherent fluorescently tagged OEC, coexpressing the endothelial surface marker CD31, in the intima of specimens from recipients of SA OEC (Fig. 3C, 3D; Supporting Information Fig. S4). In a separate series of experiments, OEC were transfused into CD1 mice 6 hours after induction of hind-limb ischemia by ligation of the femoral artery; limb ischemia was confirmed immediately after surgery using laser Doppler, with the sham operated contralateral limb serving as control. Fourteen days later, laser Doppler analysis was repeated before harvesting of the gastrocnemius muscle for confocal microscopic enumeration of engrafted fluorescently tagged OEC coexpressing CD31. Compared with mice receiving vehicle medium alone, WE OEC significantly augmented ischemic limb perfusion at 14 days, while SA OEC had no impact (Fig. 3E, 3F); numbers of engrafted CD31 expressing OEC were also significantly greater in mice receiving WE versus SA OEC (Fig. 3G, 3H; Supporting Information Fig. S5). Hence, SA OEC appeared unable to promote conduit arterial repair or ischemia induced angiogenesis, in contrast with control WE OEC.

Restoration of Akt Activity Rescues SA OEC Function In Vitro

Based upon the upstream location of Akt relative to eNOS, and literature demonstrating the critical role of both molecules in angiogenesis [19–21], we elected to augment OEC Akt activity in SA OEC using lentiviral vector gene delivery of a constitutively active Akt1 mutant (E17KAkt—Supporting Information Fig. S6A) [22, 23]. Self-inactivating lentiviral vectors (LVHVSLS2 and LVSinCSGWdlNot1) were used to introduce E17KAkt or EGFP (control), with viral titers chosen to augment SA OEC phospho-Akt S473 content approximately threefold, to achieve levels comparable to control European OEC (Supporting Information Fig. S6B). E17KAkt expressing SA OEC exhibited increased phospho-Akt S473 content and Akt activity as measured using a cell free GSK phosphorylation assay (Fig. 4A, 4B); Akt activity in EGFP expressing and native SA OEC was comparable (Fig. 4B). E17KAkt expressing SA OEC also demonstrated increased total- and phospho-eNOS S1177 (Fig. 4C; Supporting Information Fig. S7A); this was associated with augmented in vitro VEGF induced angiogenesis, a phenomenon which could be completely blocked by the NOS antagonist L-NMMA (Fig. 4D). Moreover, E17KAkt expressing SA OEC demonstrated markedly enhanced survival in a simulated ischemic environment, comprising of hypoxia, serum deprivation, oxidative stress, and the inflammatory mediator TNF-α (Fig. 4E). These data implicate increased Akt/eNOS signaling as contributing to the augmented function of E17KAkt expressing SA OEC in vitro. Migration of E17KAkt and EGFP expressing OEC toward VEGF were comparable (Supporting Information Fig. S7B).

Restoration of Akt Activity Rescues SA OEC Function In Vivo

Intravenous transfusion of EGFP expressing SA OEC was associated with unchanged femoral artery re-endothelialization, whereas E17KAkt expressing OEC augmented re-endothelialization to a magnitude comparable with nontransduced European cells (Fig. 5A). Confocal microscopy of injured femoral artery specimens revealed significantly greater numbers of engrafted CD31 expressing OEC in mice exposed to E17KAkt, versus EGFP, expressing SA OEC (Fig. 5B). Fourteen days after induction of hind-limb ischemia, E17KAkt SA OEC

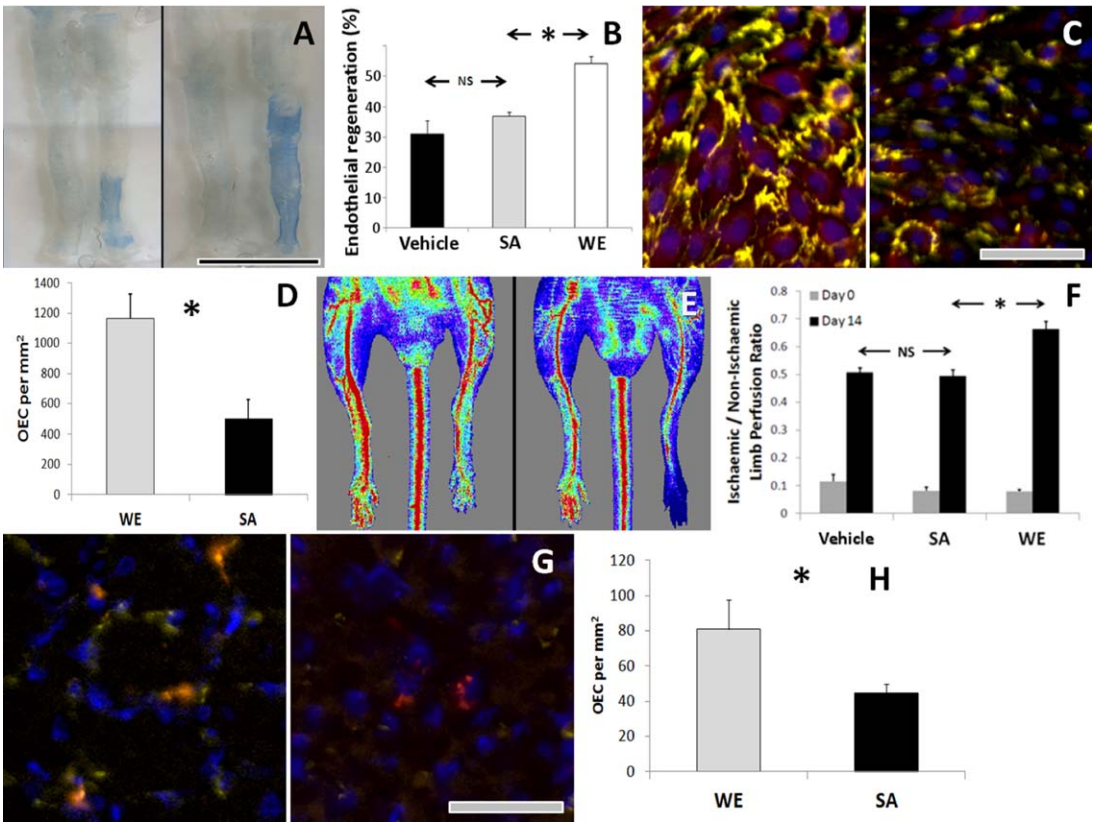

Figure 3. *In vivo* assessment of outgrowth endothelial cell (OEC) function. **(A):** Representative injured (left) and noninjured (right) femoral arteries from mice receiving white European (WE) OEC (left panel) and South Asian (SA) OEC (right panel) demonstrating greater residual endothelial denudation, indicated by Evan's blue staining, in the injured vessel exposed to SA OEC (scale bar = 5mm); **(B)** femoral artery re-endothelialization is augmented in mice receiving WE, but not SA OEC, nor vehicle ($n = 7$ in all groups); **(C)** representative confocal microscopy of injured intima from vessels exposed to WE (left) and SA (right) OEC, demonstrating cell engraftment and CD31 coexpression (Scale bar = 50 μm; blue—DAPI, red—CMDiI cell tracker-labeled OEC; Yellow—Chromeo 642 anti-CD31); **(D)** greater numbers of OEC engraft in vessels exposed to WE than SA OEC ($n = 5$ per group); **(E)** representative laser Doppler flux images (day 14 post-operatively) from mice receiving WE (left) and SA (right) OEC after induction of limb ischemia; **(F)** limb blood flow, while comparable post-operatively, is greater on day 14 in mice receiving WE OEC than those receiving vehicle or SA OEC ($n = 5$ per group); **(G)** representative confocal microscopy of ischemic muscle from mice receiving WE (left) and SA (right) OEC, showing cell engraftment and CD31 coexpression (white arrows) (scale bar = 50μm; blue—DAPI; red—CMDiI cell tracker-labeled OEC; purple—Chromeo642 anti-CD31); **(H)** greater numbers of OEC engraft in ischemic muscle exposed to WE than SA OEC ($n = 5$ per group). All data are displayed as mean ± SEM; *, $p < .05$; $p \geq .05$ is denoted by nonsignificant. Enlarged versions of panels (C) and (G), with separation of color channels, are presented in the Supporting Information figures. Abbreviations: NS, nonsignificant; OEC, outgrowth endothelial cell; SA, South Asian; WE, white European.

also demonstrated a restored capacity to augment ischemic limb perfusion, in contrast to EGFP expressing SA OEC (Fig. 5C; Supporting Information Fig. S8). Ischemic limb musculature exposed to E17KAkt expressing SA OEC also demonstrated increased numbers of engrafted CD31 OEC, when compared with control mice receiving EGFP expressing SA OEC (Fig. 5D). Hence, augmenting Akt activity in SA OEC is associated with restored capacity of infused progenitors to promote vascular repair and regeneration.

Silencing of Akt1 in WE OEC Recapitulates the Dysfunction Noted in SA OEC

To support the causal role of Akt1 in our preceding observations, we went on to silence Akt1 in OEC derived from WE men. In comparison with scrambled siRNA transfected controls, Akt1 siRNA transfected OEC exhibited a mean 51% reduction in Akt1 protein (Fig. 6A), broadly reproducing the reduction in Akt1 noted in SA OEC. This was associated with markedly reduced migration toward VEGF in Boyden chamber apparatus (Fig. 6B). When infused into CD1 mice after wire-induced femoral artery injury, Akt1 knockdown OEC less effectively promoted re-endothelialization (Fig. 6C), and engrafted in markedly reduced numbers (Fig. 6D).

Discussion

Our data are the first to demonstrate the dysfunction of OEC derived from apparently healthy South Asian men using a detailed array of in vitro and in vivo assessments; furthermore, these functional deficits have been linked to molecular abnormalities relevant to vascular repair. In particular, by demonstrating improved SA OEC function after lentiviral augmentation of Akt activity, we have causally implicated this molecule in the OEC dysfunction related to South Asian ethnicity. Further support is provided by the observation that silencing of Akt1 in WE OEC mimics the dysfunction of SA OEC, both in vitro and in vivo. Moreover, our work provides the first demonstration of rescued in vivo OEC function in

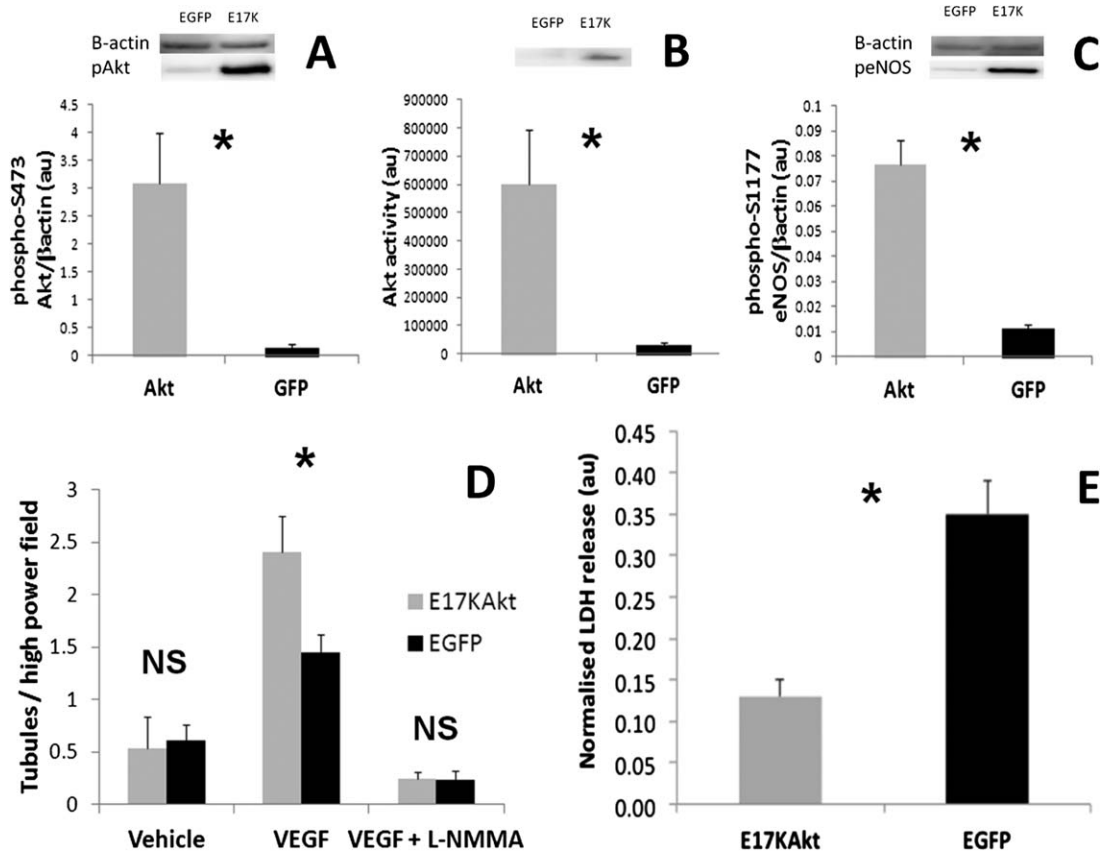

Figure 4. In vitro assessment of outgrowth endothelial cell (OEC) expressing constitutively active Akt. **(A):** Phospho-S473 Akt content is increased in E17K expressing cells, relative to EGFP expressing, or untreated South Asian (SA) OEC (representative immunoblots included; $n = 6$ in all studies); **(B)** Akt activity is increased in E17K expressing cells, relative to EGFP expressing or untreated SA OEC (representative immunoblots included; $n = 6$ in all studies); **(C)** phospho-S1177 endothelial nitric oxide synthase (eNOS) is increased in E17K expressing cells, relative to EGFP expressing or untreated SA OEC (representative immunoblots included; $n = 6$ in all studies); **(D)** SA OEC expressing E17KAkt, but not EGFP, demonstrate augmented vascular endothelial growth factor (VEGF) induced in vitro angiogenesis, which is completely abrogated by antagonism of NOS with L-N^G-nitro-L-arginine methyl ester; **(E)** SA OEC expressing E17KAkt exhibit greater survival than paired SA OEC expressing EGFP when exposed to a simulated ischemic environment ($n = 4$ per group). All data are displayed as mean ± SEM; $p < .05$ is denoted by *. Abbreviations: EGFP, enhanced green fluorescent protein; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; NS, nonsignificant; VEGF, vascular endothelial growth factor.

cells derived from subjects at increased risk of major cardiovascular events, a potentially important step in defining future cardiovascular repair therapies.

A growing body of evidence supports the elevated cardiovascular risk associated with South Asian ethnicity, compared with European ethnicity [4, 24, 25]; insulin resistance and diabetes have been strongly implicated in this phenomenon [25]. Our previous work has demonstrated important abnormalities in the vascular biology of apparently healthy, but insulin resistant, South Asian men [8, 9]. Endothelial dysfunction/damage were suggested by impaired flow mediated vasodilatation and elevation of circulating endothelial microparticles, while marked reductions in circulating progenitor subsets were apparent. Furthermore, physiological mobilization of progenitors (the $CD34^+/CD45^-$ fraction of which contain OEC precursors) [10, 11] appeared reduced in SA men, as a result of reduced NO bioavailability [8]. Our current data complement and substantially advance these observations, demonstrating clear perturbation in the function of ex vivo expanded OEC, and mechanistically implicating impaired Akt signaling in this process. The causality of such impaired signal transduction in SA OEC is unclear, although abundant data support the role of Akt, and downstream eNOS, signal transduction in promoting vascular repair and abrogating atherogenesis [19–21, 26, 27]. Importantly, our data also support the capacity of E17KAkt to augment phospho-eNOS S1177, a surrogate of eNOS activity, and demonstrate that the increased in vitro angiogenesis associated with E17KAkt expression can be blocked with a NOS antagonist.

As discussed earlier, insulin resistance is thought to be a major contributor to the increased cardiovascular risk noted in South Asian populations, based upon often circumstantial epidemiological and in vitro data. It is therefore interesting to speculate as to whether insulin resistance could underlie the reparative dysfunction noted in OEC derived from SA men. Certainly, impaired Akt/eNOS signal transduction is defining characteristic of vascular insulin resistance, and we have demonstrated this phenomenon here in OEC, and previously in healthy South Asian men using vascular ultrasound studies [8]. Moreover, we have previously demonstrated in mice haplo-insufficient for the insulin receptor, that globally reduced insulin signaling is associated with reduced vascular

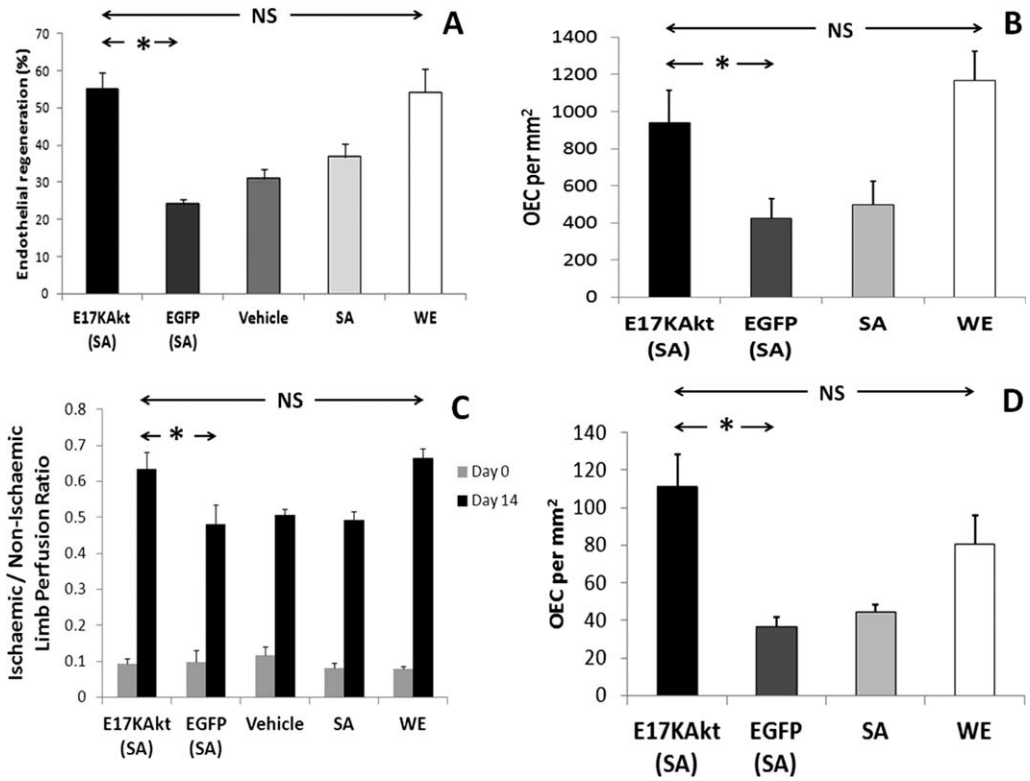

Figure 5. In vivo assessment of outgrowth endothelial cell (OEC) expressing constitutively active Akt. () Expression of E17KAkt, but not EGFP, in South Asian (SA) OEC rescues capacity to re-endothelialise injured femoral artery, when compared with unmodified white European (WE) OEC ($n = 6$ in E17KAkt and EGFP groups and $n = 7$ in all other groups). **(B):** Engraftment of OEC into the injured femoral artery is significantly increased in SA OEC expressing E17KAkt versus EGFP ($n = 5$ per group); **(C)** despite similar post-operative limb-ischemia, perfusion recovery 14 days later is only augmented in mice receiving SA OEC expressing E17KAkt, not EGFP ($n = 5$ per group); **(D)** engraftment of OEC into the ischemic gastrocnemius muscle is significantly increased in SA OEC expressing E17KAkt, versus EGFP ($n = 5$ per group). All data are displayed as mean ± SEM; *, $p < .05$. Abbreviations: EGFP, enhanced green fluorescent protein; NS, non-significant; OEC, outgrowth endothelial cell; SA, South Asian; WE, white European.

repair potentially as a result of defects in circulating progenitor cell function [15]. Ultimately however, whether the signaling abnormalities noted in SA OEC are due to insulin resistance, ethnicity, or both, is impossible to ascertain in human studies, due to technical and ethical considerations.

Interestingly, other published data using a murine model of myocardial infarction suggest that the reparative potential of OEC derived from healthy volunteers can be augmented by combined over-expression of wild-type Akt1 and heme-oxygenase-1 [14]. In agreement with our data, this work may suggest that Akt plays an important as a survival factor for OEC, which are likely to be exposed to noxious stimuli in ischemic tissues undergoing repair. Moreover, their data concurs with our observations regarding the beneficial impact of Akt upon OEC mediated angiogenesis in vivo. Our data advance these observations by showing that it is possible to rescue the diminished reparative capacity of OEC derived from people in a group at high risk of cardiovascular events.

OEC are recognized as representing an attractive form of autologous cell based cardiovascular repair therapy, given their close mimicry of endothelial phenotype and capacity for significant ex vivo expansion and manipulation [1]. Indeed, they have already been demonstrated to augment myocardial angiogenesis and stimulate beneficial left ventricular remodeling in large animal models of myocardial infarction [28]. Hence, these cells may offer the prospect of preventing or retarding cardiovascular morbidity in groups of patients at high risk of adverse events. As far as we are aware, our data is the first to demonstrate restoration of dysfunctional human OEC mediated vascular repair in vivo, an important proof of principle for human autologous cell based vascular repair therapy. Equally, our observations may be relevant to the development of pharmacological strategies to promote endogenous vascular repair in South Asian populations, which may even be relevant to insulin resistant populations in other ethnic groups. However, this goal may be more effectively achieved by first identifying the cause(s) of reduced Akt content and signaling in SA OEC—this will be an important goal of future studies.

While our data offer important insights into the mechanisms underlying abnormal indices of vascular repair in South Asian men, it is important to acknowledge some limitations. First, human cell-based cardiovascular repair therapy remains relatively nascent, with bone marrow-derived mononuclear cells being the only fraction to have been studied in any detail (and data from large phase III studies are awaited [29]). Many other cell-sources have been applied in small phase I trials, although these give little meaningful indication of therapeutic efficacy [30]; OEC have not yet been administered to humans, but large animal studies suggest a greater efficacy than mesencyhmal stem cells in promoting recovery after

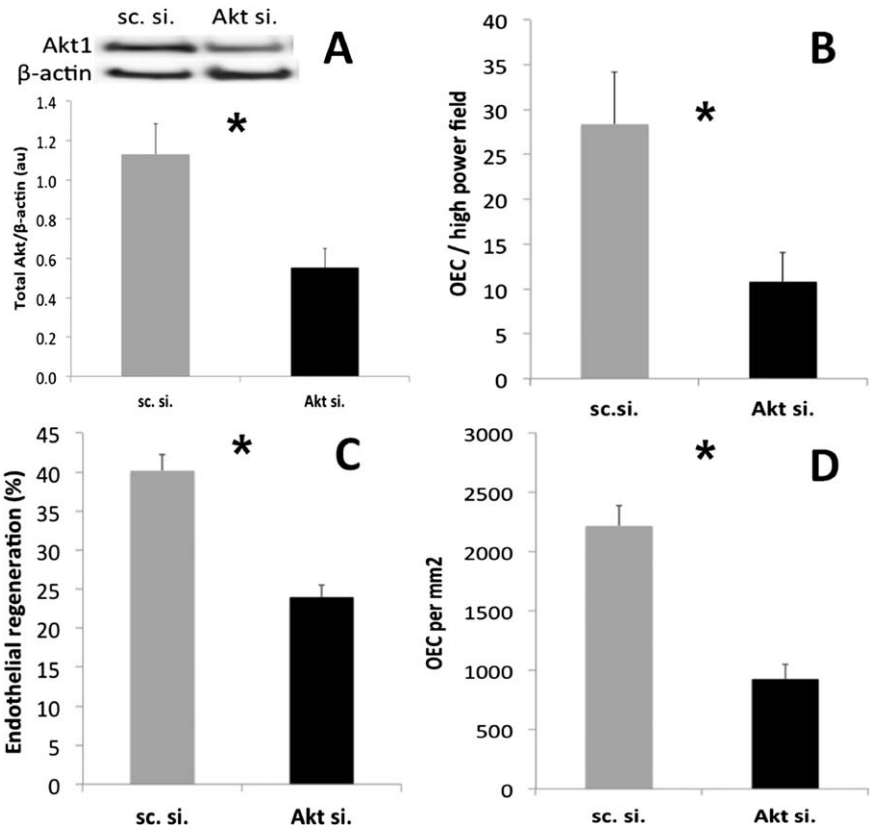

Figure 6. Akt1 silencing in white European (WE) outgrowth endothelial cell (OEC) recapitulates the functional abnormalities noted in South Asian (SA) OEC. **(A):** Akt1 expression was reduced by a mean of 51% in WE OEC exposed to Akt1 siRNA (Akt si.) versus paired control samples exposed to scrambled siRNA (sc. si.)($n = 6$ per group); **(B)** silencing of Akt1 reduced migration toward vascular endothelial growth factor (VEGF) ($n = 5$ per group); **(C, D)** when infused after femoral arterial injury, Akt1 silenced OEC were less able to promote re-endothelialisation **(C)** and engrafted less frequently into the injured vessel intima **(D)** ($n = 6$ per group). All data are displayed as mean ± SEM; *, $p < .05$. Abbreviation: OEC, outgrowth endothelial cell.

myocardial infarction [28]. The capacity to rescue the reparative properties of dysfunctional autologous progenitors has been raised as an important obstacle to clinical translation of such therapies [31]; in this regard, our investigation provides an important proof of principal in OEC. As discussed earlier, cell based therapies may not be the only means of promoting vascular repair—our data may also suggest avenues of further research into its pharmacological manipulation. However, chronic over-activation of Akt has also been linked with diminished indices of vascular repair [32, 33], and so it may desirable to transiently augment vascular or OEC Akt activity when vascular repair and regeneration is most desired. By understanding why Akt signaling is impaired in SA OEC, it may also be possible to develop more targeted therapies which circumvent this issue.

Conclusion

In conclusion, we have demonstrated that OEC derived from healthy South Asian men exhibit significant blunting of vascular reparative function in vitro and in vivo, which is mechanistically linked to reduced Akt/eNOS signaling. By augmenting Akt signaling using a lentiviral vector, we have provided the first ever demonstration that dysfunction of OEC from humans at increased cardiovascular risk can be corrected.

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Author Contributions

R.M.C.: conception and design, provision of study materials or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; N.Y.Y., H.V., B.N.M., V.B., A.S., R.S.M., A.M.N.W., A.B., M.A.B., S.G., H.I., M.C.G., M.R., and J.L.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; S.L.S.: provision of study materials or patients, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; J.A.: provision of study materials or patients, collection and/or assembly of data, final approval of manuscript; P.S.: collection and/or assembly of data, data analysis and interpretation; final approval of manuscript; K.E.P., S.P., S.B.W., and D.J.B.: conception and design, final approval of manuscript; M.T.K.: conception and design, provision of study materials or patients, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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