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NOX4 is a major regulator of cord blood-derived endothelial colony-forming cells which promotes post-ischaemic revascularization

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Aims
Cord blood-derived endothelial colony-forming cells (CB-ECFCs) are a defined progenitor population with established roles in vascular homeostasis and angiogenesis, which possess low immunogenicity and high potential for allogeneic therapy and are highly sensitive to regulation by reactive oxygen species (ROS). The aim of this study was to define the precise role of the major ROS-producing enzyme, NOX4 NADPH oxidase, in CB-ECFC vasoreparative function.

Methods and results
In vitro CB-ECFC migration (scratch-wound assay) and tubulogenesis (tube length, branch number) was enhanced by phorbol 12-myristate 13-acetate (PMA)-induced superoxide in a NOX-dependent manner. CB-ECFCs highly-expressed NOX4, which was further induced by PMA, whilst NOX4 siRNA and plasmid overexpression reduced and potentiated in vitro function, respectively. Increased ROS generation in NOX4-overexpressing CB-ECFCs (DCF fluorescence, flow cytometry) was specifically reduced by superoxide dismutase, highlighting induction of ROS-specific signalling. Laser Doppler imaging of mouse ischaemic hindlimbs at 7 days indicated that NOX4-knockdown CB-ECFCs inhibited blood flow recovery, which was enhanced by NOX4-overexpressing CB-ECFCs. Tissue analysis at 14 days revealed consistent alterations in vascular density (lectin expression) and eNOS protein despite clearance of injected CB-ECFCs, suggesting NOX4-mediated modulation of host tissue. Indeed, proteome array analysis indicated that NOX4-knockdown CB-ECFCs largely suppressed tissue angiogenesis, whilst NOX4-overexpressing CB-ECFCs up-regulated a number of pro-angiogenic factors specifically-linked with eNOS signalling, in parallel with equivalent modulation of NOX-dependent ROS generation, suggesting that CB-ECFC NOX4 signalling may promote host vascular repair.

Conclusion
Taken together, these findings indicate a key role for NOX4 in CB-ECFCs, thereby highlighting its potential as a target for enhancing their reparative function through therapeutic priming to support creation of a pro-reparative microenvironment and effective post-ischaemic revascularization.

Keywords
NADPH oxidase • NOX4 • Angiogenesis • Reactive oxygen species • Ischaemia

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1. Introduction

Angiogenesis is central to many important physiological events, such as placental growth, embryonic development, and wound-repair, whilst angiogenic dysfunction is a major pathological driver in ischaemic diseases, characterized by reduced tissue blood supply, restricted oxygen/nutrient delivery, compromised removal of metabolic waste products, cell death and inflammation.\(^1\) As management of ischaemic tissue repair/remodelling remains a clinical challenge, identification of novel cell-based therapies capable of promoting revascularization in diseased patients is the focus of much current research.\(^2\) In this regard, endothelial colony-forming cells (ECFCs), a subclass of the endothelial progenitor cell lineage, which are rapidly recruited to sites of ischaemia where they promote formation of new blood vessels and effective tissue reperfusion, hold great therapeutic potential.\(^3\) Indeed, administration of ECFCs into clinically-relevant models of experimental ischaemia (brain, limb, myocardium, retina) has highlighted a prominent role in key revascularization events.\(^3,4\) Whilst ECFCs can be isolated from the peripheral circulation of adults, they are more readily obtained from umbilical cord blood-derived ECFCs (CB-ECFCs), with these cells being highly-proliferative and easily-expanded in culture.\(^5,6\) Importantly, CB-ECFCs form more functional vessels than peripheral blood ECFCs and display lower immunogenic potential, thereby increasing their prospect for allogeneic therapy.\(^5,6\) Indeed, initial excitement surrounding autologous ECFC therapies has been largely dampened by evidently impaired functionality of cells isolated from ischaemic patients with common comorbidities,\(^7\) so focus has largely shifted towards harnessing the innate reparative capacity of allogeneic CB-ECFCs.

However, despite the clear clinical potential of CB-ECFCs, significant challenges must be addressed before they can be advanced towards a viable cell therapy for ischaemic disease. In addition to development of autologous vs. allogeneic strategies, insufficient therapeutic efficacy and suppression of ECFC functionality within diseased tissues represent major current barriers to translation. Therefore, much recent attention has focussed on identifying mechanisms underlying the vasoreparative functions of ECFCs and investigating the influence of the disease microenvironment (e.g. hypoxia, pro-inflammatory), towards modulation for therapeutic benefit. For example, pre-conditioning of cells prior to administration to target and enhance particular pro-angiogenic pathways, co-treatment with mesenchymal stem cells, and identification of highly proliferative sub-populations for enrichment, have all been explored as potential approaches to improve in vivo ECFC functionality. Targeting of reactive oxygen species (ROS)-producing enzymes is a particularly attractive option given that these proteins are implicated in numerous intracellular signalling pathways linked with angiogenesis.\(^10\) Indeed, ECFCs appear to be more resistant to oxidative stress compared with myeloid-derived and circulating early endothelial progenitors,\(^11\) which may underlie their superior in vivo function. Notably, an in vitro role for NOX4 NADPH oxidase, the most prevalent NOX isofrom in endothelial cells (ECs), has been reported in ECFCs derived from saphenous vein and mammary artery of patients undergoing coronary artery bypass grafting. NOX4 was specifically implicated in regulating proliferative and migratory responses within these ECFCs, whilst also conferring protection against cell death, although neither detailed signalling nor in vivo analyses were performed.\(^12\)

In this study, we sought to further examine the role of NOX4-dependent ROS signalling as a major determinant of the functional/ reparative capabilities of ECFCs, specifically studying cells isolated from human umbilical cord blood, as a more proliferative and therapeutically-relevant source. CB-ECFCs were initially confirmed to display superior migratory and tube/barrier formation properties compared with mature ECs, whilst treatment with phorbol 12-myristate 13-acetate (PMA) to induce endogenous superoxide, potentiated in vitro CB-ECFC function in a NOX-dependent manner. Notably, NOX4 was the most highly expressed isoform in CB-ECFCs (with NOX1, NOX2, and NOX5 present at low levels) and was the only NOX up-regulated with PMA treatment, whilst targeted NOX4 knockdown (KD) and overexpression (OE) led to reduced and enhanced migratory and vascular network-generating capabilities, respectively, highlighting a major regulatory role in vitro. NOX4-KD CB-ECFCs injected into mouse ischaemic hindlimbs demonstrated reduced ability for revascularization, whilst NOX4-OE CB-ECFCs promoted reperfusion and vascular remodelling via establishment of a pro-angiogenic host microenvironment associated with increased functional vasculature and eNOS signalling. Taken together, these data clearly support an important and novel role for NOX4 in regulation of CB-ECFC function and signalling, whilst highlighting the intriguing possibility for in vivo priming of allogeneic CB-ECFCs by selective targeting of NOX4 to increase their cytotherapeutic potential for human ischaemic cardiovascular disease.

2. Methods

A detailed methods section is available in the Supplementary material online. In brief, CB-ECFCs (obtained from multiple donors) were immunophenotyped by flow cytometry prior to NOX siRNA KD or plasmid OE. In vitro function was assessed by scratch-wound or matrigel tubulogenesis assays in the presence/absence of PMA, tert-butyl hydroperoxide (tBuH), Polyethylene Glycol-Catalase (PEG-CAT), Polyethylene Glycol-Superoxide Dismutase (PEG-SOD), and VAS2870 to interrogate ROS-specific signalling, and barrier integrity determined using an xCelligence system, with comparison to mature human aortic ECs (HAoECs). In vivo function of NOX4-modified CB-ECFCs was assessed by injection (5 × 10^5) in to ischaemic hindlimbs of immunocompromised NOD.CB17- Prkdcscid/Il2rgCrl mice induced by permanent ligation of the left femoral artery. CB-ECFC persistence was assessed using DiR-fluorescent labelling and imaging, whilst hindlimb perfusion was quantified at 7 days by laser Doppler imaging prior to sacrifice by cervical dislocation at 14 days. mRNA and protein expression in both CB-ECFCs and tissue were analysed by conventional or qRT-PCR and western blot, respectively, with normalization to ACTB or HPRT, whilst a proteome array was used to assess modulation of host angiogenic signalling by injected CB-ECFCs. ROS production by CB-ECFCs was quantified by 2′,7′-dichlorofluorescin (DCF) and dihydroethidium (DHE) fluorescence in the presence of PEG-CAT or PEG-SOD with detection by flow cytometry or confocal microscopy, respectively. All animal procedures were performed in accordance with the guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK) and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Queen’s University Belfast Animal Welfare and Ethical Review Body. Statistical analysis was performed by one-way analysis of variance with Bonferroni or Dunnett’s post hoc testing or unpaired Student’s t-test.

3. Results

3.1 CB-ECFCs display superior migratory capacity and tubulogenic function vs. mature ECs in vitro

CB-ECFCs used in this study formed a cobblestone-like monolayer in culture, typical of ECs, and did not differ phenotypically from mature
HAoECs (Figure 1A). Consistent with their recognized profile, flow cytometry immunophenotyping confirmed that each CB-ECFC clone highly expressed CD31 and CD105 (EC markers) whilst showing low expression of CD90 (mesenchymal marker) and negative expression of CD45 (haematopoietic marker; Figure 1B, Supplementary material online, Figure S1A). To establish that our CB-ECFCs possessed superior functional capacity relative to HAoECs, assays were conducted in vitro for direct comparison. As expected, CB-ECFCs displayed enhanced migration ability vs. HAoECs in scratch-wound experiments, as indicated by increased migration area (Figure 1C). Following seeding of both cell types in matrigel, CB-ECFCs also demonstrated improved capacity to form vascular networks in vitro, as indicated by increased tube length and branch number (Figure 1D). Furthermore, in experiments conducted using an RTCA xCELLigence system, CB-ECFCs formed a tighter and less ‘leaky’ barrier compared with HAoECs, as indicated by increased electrical impedance, quantified as mean cell index and area under the curve (Figure 1E). Taken together, these data clearly indicate a greater capacity for angiogenic function in CB-ECFCs under basal conditions vs. mature HAoECs, supporting their evident therapeutic potential.

3.2 Pro-migratory function of CB-ECFCs is dependent upon and stimulated by low levels of endogenous superoxide in vitro

Having established increased capacity for migration and vessel formation in CB-ECFCs vs. mature ECs, we sought to investigate the effect of oxidative signalling on these in vitro functions. PMA, which canonically activates protein kinase C to stimulate endogenous superoxide, and tBH, an exogenous hydrogen peroxide (H₂O₂) mimic, were used as pro-oxidative stimuli (not to specifically activate NOX4). Preliminary experiments found that 100 nmol/L PMA was sufficient to induce CB-ECFC superoxide generation, which was suppressed at higher concentrations (Supplementary material online, Figure S1B). Similarly, scratch-wound assays indicated that 100 nmol/L PMA (but not 200 nmol/L) increased CB-ECFC migration when cultured in phenol red-free DMEM; cell migration was not stimulated by PMA in Endothelial Growth Medium-2 (EGM2) even at higher concentrations up to 1 μmol/L, likely due to the presence of antioxidants and growth factors in this media (e.g. ascorbic acid, vascular endothelial growth factor (VEGF)).

Figure 1 CB-ECFCs display superior migratory capacity and tubulogenic function vs. mature ECs in vitro. (A) Representative images of HAoECs and CB-ECFCs (scale bar 100 μm). (B) Representative flow cytometry immunophenotyping to confirm absence of haematopoietic and mesenchymal markers (CD45, CD90), and presence of EC markers (CD105, CD31; n = 3, data collected from three clones, % positive cells for each marker is shown in the top left of each panel). (C) Scratch-wound assay to determine migration area over 16 h in HAoECs (n = 6) and CB-ECFCs (n = 18, combined data from three clones); representative images shown from a single clone. Scale bar 150 μm. (D) Matrigel tubulogenesis assay over 24 h in HAoECs (n = 6) and CB-ECFCs (n = 9, combined data from three clones) with quantification of tube length/branch number; representative images shown from a single clone. Scale bar 100 μm. (E) Barrier formation assay, based on impedance (cell index) measurements over 20 h using an RTCA xCELLigence system; mean cell index across all time-points is shown together with calculated area under the curve for both CB-ECFCs and HAoECs (n = 6, data collected from two CB-ECFC clones, data for one representative clone presented). For all scatter plots, data are mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student’s t-test.
leading to a suppressed pro-oxidative environment and therefore migratory response (Supplementary material online, Figure S1C). It should be noted, however, that basal migration was lower in DMEM due to loss of growth factors and additional stimulatory factors present in EGM2. Given these preliminary findings, to effectively interrogate the effect of oxidative signalling, CB-ECFCs were treated with 100 nmol/L PMA in DMEM (16–24 h) in all subsequent experiments. Although migration was increased following treatment of cells with PMA, exogenous tBH did not alter this in vitro function (Figure 2A). Furthermore, both basal and PMA-stimulated migration were completely ablated by the superoxide scavenger, PEG-SOD (100 U/mL), highlighting the critical importance of superoxide signalling in CB-ECFCs under both normal and stimulated conditions. In contrast, co-treatment of CB-ECFCs with the H2O2 scavenger, PEG-CAT (500 U/mL), had no effect on either basal or tBH-stimulated cell migration (Figure 2A). Indeed, tBH treatment caused a catastrophic collapse in the cells’ abilities to form tubes in matrigel, which could not be reversed by PEG-CAT (Figure 2B), highlighting an apparently detrimental role for exogenous H2O2 signalling in this setting. In contrast, basal tube formation was reduced in the presence of PEG-CAT (Figure 2B) indicating positive effects of endogenous H2O2 on CB-ECFC function. Similar to the scratch-wound assays, PMA promoted tubulogenesis of CB-ECFCs in matrigel, as indicated by increased branch number, an effect which was almost completely ablated by PEG-SOD (Figure 2C). Taken together, these data clearly indicate that CB-ECFC in vitro function is regulated by low levels of endogenous ROS, with superoxide, rather than H2O2, appearing to exert a positive impact on their functionality.

### 3.3 NOX oxidase signalling plays an important role in CB-ECFC function in vitro

Although these data highlight differential effects of ROS (superoxide, H2O2) on CB-ECFC pro-angiogenic function, thereby indicating key involvement of redox signalling, they do not link this to specific ROS sources. In this regard, further experiments were conducted in which CB-ECFCs were treated with the pan-NOX inhibitor, VAS2870, to determine the contribution of NOX, which have been implicated in regulating progenitor cell function. Indeed, co-incubation of CB-ECFCs with VAS2870 completely ablated the pro-migratory effect of PMA although basal migration remained unaltered vs. control (Figure 3A). Similarly, in a more physiologically-relevant matrigel tubulogenesis assay, VAS2870 reduced PMA-enhanced tube formation (tube length, branch number) to below control levels, whilst also decreasing basal tubulogenesis vs.

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**Figure 2** Pro-migratory function of CB-ECFCs is dependent upon and stimulated by low levels of endogenous ROS in vitro. (A) Scratch-wound assay in CB-ECFCs over 16 h in phenol red-free DMEM to determine migration area (CTL/C; control) when treated with pro-oxidant PMA (P; 100 nmol/L) or tBH (T; 100 nmol/L) with/without PEG-SOD (S; 100 U/mL) or PEG-CAT (C; 500 U/mL) (n = 6–9, combined data from three CB-ECFC clones); representative images shown from a single clone for each treatment group. Scale bar 150 μm. (B) Matrigel tubulogenesis assay over 24 h with tBH and/or PEG-CAT treatment as in A, with quantification of tube length/branch number for control and PEG-CAT (n = 6, combined data from three CB-ECFC clones); no detectable tube formation (ND) was evident following addition of tBH (n = 6). Representative images shown from a single clone for each group. (C) Matrigel tubulogenesis assay over 24 h with PMA and/or PEG-SOD treatment as in A, with quantification of tube length/branch number (n = 5–8, combined from three CB-ECFC clones); representative images shown from a single clone for each group. Scale bar 100 μm. For all scatter plots, data are mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Bonferroni post hoc testing.
**Figure 3** NOX signalling plays an important in vitro role in CB-ECFCs. (A) Scratch-wound assay in CB-ECFCs over 16 h in phenol red-free DMEM to determine migration area (CTL/C; control) when treated with pro-oxidant PMA (P, 100 nmol/L) and/or VAS2870 (VAS/V, 100 nmol/L) (n = 5–6, combined data from three CB-ECFC clones); representative images shown from a single clone for each group. Scale bar 150 μm. (B) Matrigel tubulogenesis assay over 24 h with PMA and/or VAS2870 treatment as in A, with quantification of tube length/branch number (n = 6, combined data from three CB-ECFC clones); representative images shown from a single clone. Scale bar 100 μm. (C) Microarray data (Illumina WG-6 v3.0 expression Beadchip, n = 3 clones) for all NOX isoforms accessed at ARRAYEX-PRESS database with accession number E-MTAB-1388. (D) RT-PCR validation (25 cycle threshold, n = 3 clones) of microarray data (+ controls: NOX5-testis, NOX1-colon, NOX2-brain, NOX4-kidney; RT = no template DNA control). ACTB is shown as reference loading control. (E) Western blotting analysis of NOX1, NOX2, NOX4, and NOX5 protein expression in CB-ECFCs (n = 3 clones), ACTB is shown as reference loading control. (F) qRT-PCR analysis of NOX4, HMOX1, and eNOS in control and PMA-treated CB-ECFC samples (relative to HSP90AB1; n = 9 for NOX4, n = 7/8 for HMOX1; n = 5/6 for eNOS, combined data from three clones). For all scatter plots, data are mean ± SEM; **P < 0.01, ***P < 0.001, one-way ANOVA with Bonferroni post hoc testing (A, B), unpaired Student’s t-test (F).
controls (Figure 3B), thereby highlighting the apparent importance of intact NOX signalling to CB-ECFC functionality in vitro. Although these experiments allow direct linkage of our observed effects of endogenous oxidative stimulation to NOX signalling, as VAS2870 is non-specific they do not permit identification of the specific isoform(s) involved. Therefore, mRNA and protein expression of all four endothelial NOX isoforms (NOX1, NOX2, NOX4, NOX5) was assessed. Microarray analysis revealed NOX4 to be the most highly expressed isoform in CB-ECFCs, with low levels of NOX1, NOX2, and NOX5 detected (Figure 3C). These data were confirmed across all three clones used in this study by conventional RT-PCR, set to a cycle cut-off of 25, to define biologically-relevant levels of NOX isoforms, with only NOX4 mRNA detected (Figure 3D). Western blotting confirmed presence of NOX4 protein in addition to lower expression of NOX2, whilst NOX1 was not detected and NOX5 was present at very low levels (Figure 3E). Further supporting the possibility of NOX-dependent functions (both basally and following pro-oxidative stimulation), qRT-PCR mRNA analysis indicated that key downstream genes associated with ROS scavenging (heme oxygenase 1, HMOX1) and EC function (endothelial nitric oxide synthase, eNOS) were up-regulated following PMA treatment in parallel with induction of NOX4 (Figure 3F), underlining its potential importance in CB-ECFCs. None of the other NOX isoforms were altered in response to PMA (qRT-PCR, data not shown).

3.4 NOX4 specifically regulates angiogenic function of CB-ECFCs in vitro

Having identified NOX4 as the major isoform underlying NOX signalling in CB-ECFCs, we sought to manipulate its expression to specifically examine effects on *in vitro* cell function. Transient KD of NOX4 transcription, using commercially-obtained pooled siRNA against NOX4, was performed with maximal KD of approximately 90% of basal mRNA levels (vs. scrambled non-targeted siRNA control; SCR) at 24 h confirmed by conventional and qRT-PCR (Figure 4A) further to an initial time course experiment (data not shown). Maximal reduction of NOX4 protein was observed at 72 h (Figure 4A) and was therefore considered as the most appropriate time-point for all subsequent assays. Consistent with our VAS2870 data, basal cell migration was reduced in CB-ECFCs transfected with NOX4-targeted siRNA vs. SCR controls (Figure 4B), whilst *in vitro* angiogenesis (quantified as 3D tube area) of NOX4KD cells in matrigel was impaired (Figure 4C), strongly suggesting a key role for NOX4 in regulation of normal CB-ECFC angiogenic function. Interestingly, the reduced ability of NOX4KD cells to migrate and form tubes in vitro occurred independent of a change in total ROS generation, as assessed by DCF (Figure 4D) and DHE fluorescence (Figure 4E; Supplementary material online, Figure S3B). As low level NOX2 protein expression was found in CB-ECFCs (Figure 3E), which was increased in NOX4KD cells (Figure 4C), the effect of both NOX2KD and combined NOX2/NOX4KD on ROS generation and *in vitro* angiogenesis was also assessed. Using a commercially-obtained pooled siRNA against NOX2, reduced protein expression was confirmed at 72 h post-siRNA introduction (Supplementary material online, Figure S3A), which either alone or in combination with NOX4-targeted siRNA coincided with decreased ROS production which tended to be reduced by both PEG-SOD and PEG-CAT (Figure 4E–F, Supplementary material online, Figure S3C). Notably, NOX4KD and combined NOX2/NOX4KD were found to attenuate in vitro angiogenesis to a similar extent (Figure 4C), which was unaltered by NOX2KD (Figure 4C), indicating a likely compensatory response of NOX2 in NOX4KD cells, which was able to normalize CB-ECFC ROS generation but not in vitro function.

3.5 NOX4 overexpression promotes *in vitro* angiogenic function of CB-ECFCs

To determine if induction of NOX4 signalling could improve in *vitro* functionality of CB-ECFCs, a pcDNA4/TO-myc expression plasmid containing a full-length copy of NOX4 cDNA was introduced by electroporation (pcDNA4/TO/NOX4-myc-His). Western blotting confirmed that NOX4OE reached maximal levels (~10-fold) at 72 h post-electroporation (Figure 5A), which was therefore considered as the most appropriate time-point for all subsequent assays. In contrast to siRNA KD studies, NOX4OE promoted in *vitro* CB-ECFC function. Both cell migration (Figure 5B) and angiogenesis, quantified as 3D tube area (Figure 5C), were increased vs. empty pcDNA4/TO/mcy-His A plasmid controls. Representative images clearly indicate that NOX4OE CB-ECFCs produced both a larger and more elaborate network of endothelial tubes vs. empty vector (EV)-treated cells, further supporting the suggestion that NOX4 is an important driver of *in vitro* angiogenic responses in CB-ECFCs. Notably, flow cytometry indicated increased intensity of DCF signal in NOX4OE CB-ECFCs which was completely prevented by PEG-SOD but not PEG-CAT (Figure 5D), suggesting that the apparent pro-angiogenic effects of NOX4OE are mediated via induction of ROS-dependent signalling, specifically driven by superoxide, which is consistent with our observed beneficial effects of PMA stimulation (Figure 2). Phosphorylation of VEGFR2 protein (at Try1175) remained similar between EV and OE cells (Figure 5E) indicating that increased in *vitro* functionality of NOX4OE CB-ECFCs may occur independently of VEGF signalling.

3.6 NOX4 expression in injected CB-ECFCs promotes revascularization and pro-angiogenic signalling in ischaemic tissue

Given these data clearly implicating NOX4 as a key determinant of *in vitro* CB-ECFC function, we next determined their *in vivo* functionality in a mouse model of hindlimb ischaemia to further explore NOX4 as a potential target for improving their angiogenic function. We first confirmed previous findings by demonstrating that administration of CB-ECFCs to ischaemic hindlimb tissue promoted revascularization at 7 days post-femoral artery ligation vs. PBS control, as assessed by laser Doppler imaging (Supplementary material online, Figure S2A) and lectin staining of blood vessels in ischaemic tissue (Supplementary material online, Figure S2B). As previous studies have suggested that ECFCs do not permanently integrate with the host vasculature,4,14 survival of CB-ECFCs in situ was assessed by injection of fluorescently-labelled CB-ECFCs prior to *in vivo* detection (Bruker In-vivo Extreme II). Although cell presence in ischaemic tissue was detected at both 1 and 7 days post-injection, CB-ECFCs were absent by 14 days (Figure 6A), which was substantiated by conventional RT-PCR in excised tissue at 14 days indicating lack of amplification of human leucocyte antigen (HLA), thereby confirming clearance of CB-ECFCs in injected mice (Figure 6B). Notably, rescue of tissue perfusion at 7 days was attenuated following injection of NOX4KD CB-ECFCs vs. SCR control, whilst hindlimb blood flow was markedly improved after administration of NOX4OE CB-ECFCs vs. EV-containing cells (Figure 6C). To more specifically assess effects of NOX4 modification on host vasculature, we performed western blot and immunocytochemistry analysis of eNOS and lectin, respectively. Endogenous eNOS expression was clearly elevated in OE tissue consistent with increased vessel number and/or improved function (Figure 6D), whilst eNOS levels in KD tissue tended to be reduced. Indeed, augmented vasularzation was confirmed by higher levels of lectin staining in tissue.
NOX4 regulation of cord blood-derived ECFCs

Figure 4 NOX4 specifically regulates in vitro angiogenic function of CB-ECFCs. (A) CB-ECFCs were transfected with SMARTpool® siRNA and NOX4 targeting was confirmed via conventional RT-PCR (upper left panel, n = 3, data collected from three clones, 24 h post-siRNA transfection, representative image shown), qRT-PCR (lower left panel, relative to HSP90AB1, n = 3, data collected from three clones, 24 h post-siRNA transfection), and western blotting (right panel, relative to ACTB; n = 3, representative image and quantification shown, data collected from two clones at 72 h post-siRNA transfection). (B) Scratch-wound assay to determine migration area over 16 h (n = 9, combined data from three CB-ECFC clones) in SCR (non-targeting siRNA) or KD (NOX4-targeting siRNA) treated cells; representative images shown from a single clone. Scale bar 150 µm. (C) Three-dimensional matrigel tubulogenesis assay after 72 h, with quantification of tube area, in SCR, NOX4KD, NOX2KD, and combined NOX2/NOX4KD (SMARTpool® NOX2-targeting siRNA, 72 h post-siRNA) (n ≥ 6, combined data from 2 to 3 CB-ECFC clones). Representative images shown from a single clone. Scale bar 1 mm. (D and E) Flow cytometry analysis of fluorescent DCF (ROS indicator) in SCR, NOX4KD, NOX2KD, and combined NOX2/NOX4KD cells (n ≥ 6, data collected from at least three clones with representative traces from one clone shown) in the presence/absence of PEG-SOD (100 U/mL, 30 min treatment) or PEG-CAT (500 U/mL, 30 min treatment). (F) NOX2 western blot (n = 3, representative image and quantification shown, data collected from two clones, 72 h post-siRNA transfection). ACTB is shown as reference loading control. (G) DHE staining (superoxide-specific) of SCR, NOX4 KD, and NOX2 KD cells (n = 5, data collected from two clones, representative images shown, Scale bar 500 µm). For all scatter plots, data are mean ± SEM; *P < 0.05, ***P < 0.001, unpaired Student’s t-test (A–C, G), one-way ANOVA with Bonferroni post hoc testing (D–F).

injected with OE cells vs. that injected with EV-containing CB-ECFCs (Figure 6E), whilst lectin staining in KD tissue tended to be reduced vs. SCR control, indicative of decreased vessel formation.

To determine the prolonged host tissue response to introduction of both types of gene-modified CB-ECFCs, protein was extracted from the ischaemic muscle at 14 days (when injected cells were no longer present) and run on a mouse angiogenesis proteome profiler array. Interestingly, when normalized to their relevant control, a number of endogenous proteins (24) were found to be differentially-expressed between the two treatment groups (SCR vs. KD, EV vs. OE), highlighting modulation of host angiogenic signalling (Figure 7A). Similar to our in vitro findings, injection of NOX4OE CB-ECFCs in to ischaemic hindlimbs increased expression of a number of eNOS-linked proangiogenic factors, including dipeptidyl peptidase IV (DPP IV), endoglin, heparin-binding EGF-like growth factor (HB-EGF), matrix metalloproteinase-9 (MMP-9), osteopontin, and stromal cell-derived factor 1 (SDF-1), which were oppositely down-regulated further to administration of NOX4KD CB-ECFCs (Figure 7B, left panel). Conversely, platelet factor 4 (PF4), an established anti-angiogenic protein, was down-regulated in ischaemic tissue injected with NOX4OE cells (Figure 7B, left panel), whilst PF4 was up-regulated following injection of KD cells. Analysis of the remaining 29 targets indicated unidirectional up-regulation of expression of several eNOS-regulated/pro-angiogenic factors [e.g. angiogenin, angiopoietin-1, fibroblast growth factor-1 (FGF-1), FGF-2, VEGF-B, endostatin], which was much higher (>1000 pixel change) in tissue injected with NOX4OE vs. NOX4KD CB-ECFCs (Figure 7B, right panel), further highlighting the apparent pro-angiogenic capacity of NOX4. In addition to inducing marked signalling alterations in ischaemic tissue, NOX4OE increased protein expression of NOX2 (but not NOX4; Figure 7D) in parallel with stimulation of endogenous ROS generation, as assessed by nitrotyrosine staining (Figure 7E), supporting the findings of our in vitro studies in suggesting that the pro-angiogenic effects of NOX4 are ROS-dependent. Similarly, in line with our in vitro data, neither NOX2 (or NOX4) expression nor ROS production was altered in ischaemic tissue injected with NOX4KD CB-ECFCs. Taken together, these data clearly indicate that introduction of NOX4OE CB-ECFCs in
to ischemic tissue supports creation of a host microenvironment which is favourable for revascularization.

4. Discussion

Cardiovascular ischemia remains a leading cause of global morbidity and mortality. Development of novel cyotherapeutic strategies to manage this condition has led to huge interest in potential clinical application for endothelial progenitors to promote effective revascularization and tissue repair. In particular, ECFCs represent the most promising endothelial progenitor type with a well-described functional and immunophenotype. However, given significant challenges, such as suboptimal expansion, engraftment, survival and immunogenicity, which represent major barriers to clinical translation, much recent focus has been directed towards identification of novel approaches to support ECFC function for practical therapeutic application. In this regard, we present convincing evidence that NOX4 plays an important role in supporting normal angiogenic function of allogeneic CB-ECFCs, both in vitro and in revascularization of ischemic tissue in vivo, and that induction of NOX4 in CB-ECFCs enhances their therapeutic potential by promoting host pro-angiogenic signalling.

In agreement with the typically reported ECFC immunophenotype, the cells used in this study highly expressed CD31 and CD105 (EC markers) and were largely negative for CD90 (mesenchymal marker) and CD45 (hematopoietic marker). Furthermore, they displayed superior angiogenic function (migration, tubulogenesis, barrier formation) vs. equivalent mature HAOECs in support of their inherent capacity for engraftment and revascularization of ischemic tissue. Whilst various approaches to improve ECFC function have been explored, including OE of pro-angiogenic proteins (e.g. eNOS, erythropoietin, angiotensin-converting enzyme 2), manipulation of important epigenetic modifiers, and preconditioning by hypoxic exposure, we chose to focus on ROS signalling specifically mediated by NOX as a novel target to promote CB-ECFC functionality and pro-angiogenic signalling. Initially, we found that pharmacological induction of endogenous superoxide generation using PMA promoted in vitro cell migration and tubulogenesis in CB-ECFCs together with up-regulation of NOX4, eNOS and HMOX1 mRNA. Given the established role of NO signalling in regulation of key aspects of EC function, such as vascular tone, cellular proliferation, leucocyte adhesion, and platelet aggregation, which are known to be redox sensitive, it is not surprising that PMA stimulation of endogenous superoxide in CB-ECFCs was associated with up-regulation of eNOS, which is likely to at least partly explain their increased in vitro functionality. It is particularly notable that both basal and PMA-stimulated migration and tubulogenesis of CB-ECFCs were completely ablated by PEG-SOD indicating that endogenous superoxide is critical to support in vitro functionality of these cells. Indeed, the observation that PMA promotes CB-ECFC angiogenic function and signalling highlights its potential as a preconditioning agent prior to delivery. In this context, it is interesting that expression of HMOX1, an established endogenous antioxidant, was also up-regulated, although it should be noted that HMOX1 also serves an important protective role against endothelial dysfunction via, e.g. Nr2mediated suppression of vascular inflammation, which may explain enhanced CB-ECFC function. In contrast, whilst low concentrations of H2O2 are reported to promote vascular function in vivo, it seems that H2O2 signalling in CB-ECFCs may be detrimental to cell function, at least in vitro. Our data demonstrating that exogenous treatment with the H2O2 mimic, tBH, completely prevented tube formation by CB-ECFCs are consistent with a previous report of increased apoptosis and reduced clonogenic angiogenic capacity after H2O2 exposure. Nonetheless, our observation that tubulogenesis in CB-ECFCs under basal conditions is disrupted by PEG-CAT appears to suggest that endogenous H2O2 signalling is necessary to support normal angiogenic capacity, further highlighting the complex nature of cellular redox signalling. Additional experiments indicating that both basal and PMA-stimulated CB-ECFC migration and tube formation were completely ablated by the pan-NOX inhibitor, VAS2870, highlighted specific involvement of NOX-derived superoxide and/or H2O2 generation in mediating these ROS-sensitive processes. Indeed, CB-ECFCs expressed particularly high levels of NOX4 mRNA and protein which were further increased by PMA, indicating a potentially important role in response to stress; of the other EC isoforms, only NOX2 was expressed in CB-ECFCs at any significant level and was unaltered by PMA. This is consistent with a study highlighting an important role for NOX4 in maintaining in vitro function of ECFCs isolated from saphenous and mammary vein of patients undergoing coronary artery bypass grafting, and in protecting these cells against inflammation-associated apoptosis.

The data generated in the present study using CB-ECFCs, as a more therapeutically relevant source due to their highly proliferative/functional phenotype and low immunogenic profile, underscores an important role for NOX4 in mediating the angiogenic response of this defined subset of progenitor ECs, which could be exploited to significantly enhance their vasoparative potential. Indeed, siRNA depletion of NOX4 in CB-ECFCs markedly reduced their capacity to promote basal migration and angiogenesis vs. SCR controls. Notably, these effects occurred in the absence of alteration in global ROS production or the specific contribution of superoxide or H2O2. However, it should be noted that protein expression of NOX2, the only other isoform detected in CB-ECFCs, was increased with NOX4KD, which is consistent with compensatory induction of NOX2 reported in other cells. Indeed, NOX2KD in CB-ECFCs resulted in decreased ROS, as measured by DCF and DHE fluorescence, which is likely to explain lack of reduction in ROS generation with NOX4KD, although in vitro tube formation was not affected, indicating that NOX2 is not important for basal angiogenic function. Furthermore, simultaneous KD of both NOX4 and NOX2 produced a similar effect to NOX4KD alone with regard to in vitro angiogenesis confirming that NOX4 is the key isoform in this setting. Conversely, NOX4OE at levels consistent with previous studies in ECs, markedly improved the migratory and angiogenic capacity of CB-ECFCs vs. EV controls, independently of VEGF activation, confirming the apparently supportive function of NOX4 signalling in these cells. Notably, NOX4OE was associated with increased ROS generation, which was prevented by PEG-SOD but not PEG-CAT, confirming the suggestion from our PMA studies that superoxide is the most relevant ROS to CB-ECFC angiogenic function. With specific regard to the role of NOX4, it is important to highlight that this is in marked contrast with mature ECs in which NOX4 signalling promotes vasoprotective H2O2 generation. In CB-ECFCs, administration of tBH completely inhibited their ability to form tubes in vitro, which was also reduced by PEG-CAT, indicating apparently differential effects of exogenous and endogenous H2O2 on basal function of these cells.

Having conducted detailed in vitro assessment of NOX4 signalling in CB-ECFCs, the potential clinical significance of these findings was interrogated using an established mouse model of hindlimb ischaemia, which is well characterized for the study of peripheral artery disease and therapeutic revascularization, and in which CB-ECFCs are reported to markedly improve blood flow recovery and neovascularization.
Figure 5 NOX4OE promotes in vitro angiogenic function of CB-ECFCs. (A) CB-ECFCs were electroporated with either an EV (pcDNA4/TO/myc-His) or vector containing a full-length copy of human NOX4 cDNA (pcDNA4/TO/NOX4-myc-His) (OE). Protein was harvested from EV and OE cells and levels of NOX4 determined by western blotting at 72 h post-electroporation (n = 5, representative blot shown, data collected from two clones). (B) Scratch-wound assay to determine migration area over 16 h (n = 6, combined data from three CB-ECFC clones) in EV or OE cells; representative images shown from a single clone. Scale bar 150 μm. (C) Three-dimensional matrigel tubulogenesis assay after 72 h, with quantification of tube area, in EV and NOX4OE cells (n = 6, combined data from three CB-ECFC clones); representative images shown from a single clone. Scale bar 1 mm. (D) Flow cytometry analysis of fluorescent 2',7'-dichlorofluorescein (ROS indicator) in EV and OE cells (n = 6), data collected from three clones with representative traces from one clone shown) in the presence/absence of PEG-SOD (100 U/mL, 30 min treatment) or PEG-CAT (500 U/mL, 30 min treatment). (E) Western blot detection of phospho-VEGFR2 (Try1175, Total VEGFR2 also shown), representative blot shown (n = 4). For all scatter plots, data are mean ± SEM; *P < 0.05, **P < 0.01, unpaired Student’s t-test (A–C), one-way ANOVA with Bonferroni post hoc testing (D).
Indeed, this model has been utilized in several studies focused on defining the contribution of specific ROS sources to vascular redox signaling. Importantly, and consistent with our in vitro data, blood flow recovery and revascularization was impaired in mice treated with NOX4KD cells, which demonstrated complete ablation of the established benefits of CB-ECFC administration observed in SCR control mice, thereby highlighting a key function for NOX4 in supporting normal angiogenic function in vivo. Similarly, mice injected with NOX4OE CB-ECFCs demonstrated higher reperfusion vs. EV controls (noting that basal reperfusion was reduced with plasmid vs. siRNA-transfected CB-ECFCs), indicating that activation of NOX4 signalling promotes in vivo angiogenesis. Furthermore, when these NOX4-modified CB-ECFCs were injected in to ischaemic hindlimbs, equivalent alterations in angiogenic signalling were observed in the host mouse tissue, together with increased vascularization and eNOS expression, which were evident even after the administered cells had been cleared, demonstrating evident capability of CB-ECFCs to drive chronic vasoreparative actions which are subject to potentially therapeutically-relevant regulation by NOX4. In particular, administration of NOX4-OE CB-ECFCs promoted tissue expression of key endogenous pro-angiogenic factors, including DPPIV, endoglin, HB-EGF, MMP-9, osteopontin, and SDF-1, which all either activate or are activated by eNOS signalling, and consistent with our observation that eNOS expression is induced by NOX4 overexpression in these cells both in vitro and in vivo, providing some indication of underlying mechanism. Administration of NOX4OE CB-ECFCs also suppressed endogenous production of PF4, an anti-angiogenic chemokine reported to inhibit EC proliferation and migration, and in vivo angiogenesis. Notably, even angiogenic factors which were up-regulated by both NOX4KD and OE (unidirectional changes) were clearly altered to a greater extent in NOX4OE cell injected ischaemic tissue, indicative of more potent modulation of endogenous pro-angiogenic signalling, with the most up-regulated of these proteins, angiogenin, angiopoietin-1, FGF-1, FGF-2, VEGF-B, and endostatin, also reported to regulate or be regulated by eNOS. Nonetheless, whilst it seems that induction of eNOS-

Figure 6 Injected CB-ECFCs with modified NOX4 expression differentially regulate revascularization of ischaemic tissue. (A) The left femoral artery of immunocompromised male NOD.CB17-Prkdscid/NcrCrl mice (8–12 weeks) was ligated to induce tissue ischaemia prior to intramuscular injection with 500 000 CB-ECFCs (PBS only, untreated/UNT, SCR, KD, EV, OE). CB-ECFCs were fluorescently labelled with DiR®-HCT116(7,1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodine prior to injection to allow tracking of cells in vivo. Mice were anaesthetized and imaged at 1, 7, and 14 days post-injection (Bruker In-vivo Extreme II). Fluorescent signal indicating the presence of CB-ECFCs at day 1 and 7, but not day 14 (n = 3). (B) At 14 days, conventional RT-PCR (35 cycles) confirmed lack of CB-ECFC presence due to absence of HLA DNA PCR product (+HCT116, RT-no template DNA control, ACTB is shown as reference loading control; representative image shown). (C) Reperfusion assessed by laser Doppler imaging at 7 days post-ligation with blood flow quantified as ratio in ischaemic vs. healthy control limb (n = 6 SCR and KD, n = 8 EV and OE); representative images are shown for a single animal from each group. (D) Western blot detection and densitometric quantification (normalized to HPRT as reference loading control, representative blot shown, three animals for each group) of eNOS protein expression (n = 6). (E) Immunohistochemistry analysis of lectin expression in each group with quantification of staining using Image J (SCR and KD n = 6, EV n = 7, OE n = 8); representative images are shown for a single animal from each group. Scale bar 100 μm. For all scatter plots, data are mean ± SEM; *P < 0.05, **P < 0.01, unpaired Student's t-test (C), one-way ANOVA with Bonferroni post hoc testing (D, E).
dependent signalling is central to the pro-angiogenic actions of NOX4OE CB-ECFCs, more detailed experiments targeting specific NOX4-activated genes are clearly required in order to precisely define underlying mechanisms, which are likely to involve modulation of paracrine signalling. Furthermore, and consistent with our in vitro data, injection of NOX4OE CB-ECFCs increased ROS generation in host ischaemic tissue, as indicated by elevated nitrotyrosine levels, together with induction of murine Nox2 expression, further indicating that NOX-dependent ROS signalling is important for CB-ECFC-mediated angiogenesis. Similarly, injection of NOX4KD CB-ECFCs did not affect endogenous ROS generation or host NOX isoform expression, further indicating that induction of ROS signalling may be necessary to support

Figure 7 NOX4 expression in injected CB-ECFCs induces pro-angiogenic signalling in ischaemic tissue. The left femoral artery of immunocompromised male NOD.CB17-Prkdscid/NcrCrl mice (8–12 weeks) was ligated to induce tissue ischaemia prior to intramuscular injection with 500 000 CB-ECFCs (SCR, KD, EV, OE). Tissue was harvested 14 days post-surgery and protein extracted from each group was run on individual mouse angiogenesis proteome profiler arrays. Each spot represents a target (in duplicate, side by side). Pixel density of each spot was determined using HLImage™ software (duplicate spots for each target were averaged) and KD and OE blots were normalized to their relevant control groups (SCR and EV, respectively) using an average of the three corner reference spots before expression intensity for each target was determined. (A) Venn diagram indicating differential (29; oppositely detected in KD- and OE-injected tissue) and unidirectional (24; increased or decreased in both groups) changes in protein expression. (B) Both differential (left panel) and unidirectional (right panel) protein detection as in A are presented as heat maps (produced using Microsoft Excel). Small changes in protein detection (<1000 pixels) are shown in white; green and red colouring represents increased and decreased protein detection, respectively (>1000 pixels). (C) Original proteome array blots. Differentially-expressed targets specifically linked with eNOS signalling are highlighted (n = 6, pooled). (D and E) Western blot detection and densitometric quantification of Nox2/Nox4 (the latter normalized to HPRT as reference loading control; n = 6) and nitrotyrosine (marker of ROS generation; n = 6); representative images are shown for three animals from each group. For all scatter plots, data are mean ± SEM; ***P < 0.001, one-way ANOVA with Bonferroni post hoc testing.
CB-ECFC in vivo angiogenic function. Indeed, in support of our findings, it was reported that introduction of exosomes derived from human ECFCs in to a mouse model of acute kidney injury resulted in increased oxidative stress associated with reduction of disease progression, suggesting that endogenous ROS promote revascularization of ischaemic tissue. Interestingly, one of several host angiogenic proteins whose expression was increased by injection of NOX4OE CB-ECFCs, angiopoietin-1, is known to stimulate NOX-dependent ROS generation, which may in turn regulate Ang1/Tie2 signalling, thereby promoting angiogenesis. It is therefore possible that associated increases in murine Nox2 expression could have occurred secondary to induction of angiopoietin-1. However, it is important to note that the relationship between eNOS and NOX in both injected CB-ECFCs and host tissue is likely to be complex further to established species, compartment, concentration and temporal specificity of ROS signalling.

Whilst significant previous efforts have been directed towards prolonging ECFC survival in situ, it is notable that we clearly demonstrate that although CB-ECFCs are absent from the ischaemic hindlimb 14 days after injection, NOX4OE in these cells promotes creation of a pro-angiogenic host microenvironment which persists beyond their lifetime in the tissue. Such conditions evidently provide necessary support for the generation of functional blood vessels, as indicated by increased vascularization and eNOS expression in ischaemic mouse tissue subsequent to injection of NOX4OE CB-ECFCs, thereby highlighting this major ROS-producing enzyme as a viable target to increase therapeutic efficacy of these cells. It is also possible that specific manipulation of NOX-dependent ROS signalling within likely dysfunctional tissue of recipient patients may represent a novel approach to augment the angiogenic capacity of injected CB-ECFCs towards advancing these highly proliferative and functional cells as a practical allogeneic therapy for ischaemic disease.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**

endothelial colony-forming cells is essential for functional recovery of murine isch