Reactive oxygen species regulate the quiescence of CD34-positive cells derived from human embryonic stem cells

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Aims Reactive oxygen species (ROS) are involved in a wide range of cellular processes. However, few studies have examined the generation and function of ROS in human embryonic vascular development. In this study, the sources of ROS and their roles in the vascular differentiation of human embryonic stem cells (hESCs) were investigated.

Methods and results During vascular differentiation of hESCs, CD34+ cells had quiescence-related gene expression profiles and a large fraction of these cells were in G0 phase. In addition, levels of ROS, which were primarily generated through NOX4, were substantially higher in hESC-derived CD34+ cells than in hESC-derived CD34− cells. To determine whether excess levels of ROS induce quiescence of hESC-derived CD34+ cells, ROS levels were moderately reduced using selenium to enhance antioxidant activities of thioredoxin reductase and glutathione peroxidase. In comparison to untreated CD34+ cells, selenium-treated CD34+ cells exhibited changes in gene expression that favoured cell cycle progression, and had a greater proliferation and a smaller fraction of cells in G0 phase. Thus, selenium treatment increased the number of hESC-derived CD34+ cells, thereby enhancing the efficiency with which hESCs differentiated into vascular endothelial and smooth muscle cells.

Conclusion This study reveals that NOX4 produces ROS in CD34+ cells during vascular differentiation of hESCs, and shows that modulation of ROS levels using antioxidants such as selenium may be a novel approach to increase the vascular differentiation efficiency of hESCs.

Keywords CD34 • Human embryonic stem cells • Reactive oxygen species • Selenium • Vascular differentiation

1. Introduction

Human embryonic stem cells (hESCs), which are derived from the inner cell mass of a blastocyst, have a high self-renewal capacity and the potential to differentiate into all three germ layers. hESCs are a powerful tool for investigating the molecular mechanisms underlying human embryonic development.1 In the vascular biology field, many researchers have used hESCs to study human embryonic vascular development in vitro. In terms of regenerative medicine, understanding and optimizing the differentiation of hESCs into vascular lineages should facilitate the generation of sufficient quantities of vascular cells for potential therapeutic applications.

During hESC differentiation, vascular cells, such as vascular endothelial cells (ECs) and smooth muscle cells (SMCs), are differentiated from common precursors called vascular progenitors or angioblasts. The emergence of vascular progenitors is critical during vascular differentiation of hESCs. Therefore, many studies have isolated hESC-derived vascular progenitors and characterized their molecular and cellular properties to provide insights into the poorly defined mechanism underlying human embryonic vascular development.2–4 In this regard, we previously identified genes that are differentially expressed in hESC-derived vascular progenitors by comparing the gene expression profiles of hESC-derived vascular progenitors and other differentiated cells.5 Our previous results showed that biological processes involving mitosis and biogenesis of the ribonucleoprotein complex are substantially repressed in hESC-derived vascular progenitors. In particular, genes involved in the cell cycle, chromosome segregation, and spindle

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organization are considerably down-regulated in hESC-derived vascular progenitors, suggesting that these cells do not proliferate and remain quiescent during vascular differentiation.

Cell division is highly regulated by the cellular redox and oxidation (redox) environment. The cellular redox status is influenced by the production and removal of reactive oxygen species (ROS); therefore, ROS play an important role in cell division. ROS are commonly thought to induce cell cycle arrest due to the oxidation of macromolecules. In contrast, low levels of ROS can serve as second messengers in mitogenic signalling cascades that promote cell cycle progression. This dual action of ROS as negative and positive regulators of cell division varies according to the ROS concentration, how quickly ROS are scavenged, and the cell types. Given the critical roles of ROS in cell proliferation and cell cycle arrest, we hypothesized that the quiescence of hESC-derived vascular progenitors is associated with ROS that are generated during hESC differentiation. In this study, we examined the sources and generation of ROS during vascular differentiation of hESCs and the effect of antioxidant selenium on the cell cycle of CD34+ cells containing vascular progenitors. Elucidation of these processes might contribute to the optimization of the conditions required for vascular differentiation of hESCs, which would enhance the efficiency of vascular differentiation.

2. Methods

2.1 Cell culture

H9 hESCs (Wicell Research Institute, Madison, WI, USA) were grown on mitomycin-treated mouse embryonic fibroblasts in hESC culture medium containing DMEM/F12 (Gibco, Grand Island, NY, USA) supplemented with knockout serum replacement (20%; Gibco), penicillin–streptomycin (100 U/mL; Gibco), β-mercaptoethanol (0.1 mM; Gibco), non-essential amino acid solution (1%; Gibco), and basic fibroblast growth factor (bFGF, 5 ng/mL; ProSpec, Rehovot, Israel).

2.2 Cell culture of hESCs

H9 cells were cultured on Matrigel (BD Biosciences, Bedford, MA, USA) for 2 days before human embryoid bodies (hEBs) were formed. hEBs were differentiated in medium I [DMEM/F12 containing knockout serum replacement (10%), vascular endothelial growth factor (VEGF, 10 ng/mL; R&D Systems, Minneapolis, MN, USA), bone morphogenetic protein 4 (BMP4, 10 ng/mL; ProSpec), bFGF (5 ng/mL; ProSpec), and activin-A (3 ng/mL; ProSpec)] under hypoxic conditions (3% O2) for 8 days. hEBs were then attached to a gelatin-coated dish and further differentiated for an additional 7 days in medium II [endothelial basal medium (Lonza, Walkersville, MD, USA) containing VEGF (50 ng/mL), BMP-4 (20 ng/mL), and bFGF (5 ng/mL)] under normoxic conditions. For vascular differentiation of hESCs with selenium supplementation, sodium selenite (20 or 50 ng/mL; Sigma), bone morphogenetic protein 4 (BMP4, 10 ng/mL; R&D Systems), and basic fibroblast growth factor (bFGF, 5 ng/mL; ProSpec) were added to medium I and II.

2.3 Reverse transcription polymerase chain reaction

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen) and amplified through 25–35 polymerase chain reaction (PCR) cycles with gene-specific primers. Densitometric analysis of PCR bands was performed with Image-Lab (MC3 design, Birkerød, Denmark). For quantitative real-time PCR analysis, cDNA was analysed using SYBR Green PCR master mix (Applied Biosystems, Forster City, CA, USA). Data were analysed using the ΔΔCt method and normalized against values obtained for the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All reactions were performed in triplicate. Primer sequences are listed in Supplementary material online, Table S1.

2.4 Flow cytometry analysis

To separate CD34+ and CD34− cells differentiated from hESCs, hEBs were dissociated into single cells using dissociation buffer (ReproCell, Yokohama, Japan) and dispase (Roche, Mannheim, Germany), and labelled with phycoerythrin (PE)-conjugated anti-CD34 IgG (Dako, Inc., Carpenteria, CA, USA). CD34+ and CD34− cells were purified using a fluorescence-activated cell sorting (FACS) Aria II cell sorter and CellQuest acquisition software (BD Biosciences). Controls were stained with appropriate isotype-matched non-specific IgGs. Cell surface expression of CD34, CD31, and CD34+/CD31 was analysed using an Accuri flow cytometer (BD Biosciences). Cells pretreated with human Fe3+ blocking reagent (Milenyi Biotec, Bergisch Gladbach, Germany) were labelled with fluorescence-conjugated primary IgGs against CD34 (Dako, Inc.), CD31 (BD Biosciences), or SM22a (Abcam, Cambridge, MA, USA) for 1 h at 4 °C and washed with ice-cold buffer.

2.5 Cell cycle analysis

Sorted cells were suspended in nucleic acid staining solution (Gibco) and stained with 10 μg/mL of 7-aminoactinomycin D (AAD; eBioscience, San Diego, CA, USA) and 1 μg/mL of pyronin Y (Sigma). After washing in cold PBS, cells were kept on ice during flow cytometry analysis. Quiescent cells (G0) were identified as the population with a 2N DNA content and a lower RNA content than cells in S phase.

2.6 Cell proliferation analysis

Cells were fixed in 4% paraformaldehyde solution (Gibco) and stained with 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, CA, USA). For quantitative analysis, five representative colour images were randomly acquired per sample.

2.7 Measurement of ROS

Intracellular ROS levels were measured using 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen). Briefly, cells were incubated in serum-free medium containing 20 μM H2DCFDA at 37 °C for 20 min. After washing several times, images of the cells were acquired using a fluorescence microscope (Nikon, Tokyo, Japan). The relative H2DCFDA fluorescence intensity (corrected for background fluorescence) was measured using the NIS-Elements BR 3.1 software. Fluorescence emitted from H2DCFDA was also analysed using an Accuri flow cytometer. For experiments with ROS inhibitors, cells were pretreated with N-acetylcysteine (NAC, 100 μM; Sigma), diphenylethyleniodonium (DPI, 10 μM; Sigma), N′-nitro-arginine methyl ester (L-NAME, 100 μM; Calbiochem, Billerica, MA, USA), apocynin (100 μM; Sigma), allopurinol (100 μM; Sigma), Carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrozone (FCCP, 60 μM; Sigma), auranofin (AF, 5 μM; Sigma), aurothioglucoside (ATG, 20 μM; Sigma), PX12 (50 μM; Sigma), mercaptosuccinic acid (MSA, 5 μM; Sigma), or catalase (3000 U/mL; Sigma) at 37 °C for 5–30 min. Cells pretreated with hydrogen peroxide (100 μM; Sigma) were included as positive controls. For negative controls, cells were not incubated with H2DCFDA.

2.8 Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde (Sigma), permeabilized with 0.5% Triton X-100 (Sigma), and blocked in 10% normal goat or rabbit serum (Vector Laboratories, Inc.). Cells were incubated with primary IgGs against phospho-p53 (Ser15, Cell Signaling Technology, Danvers, MA, USA), CD34 (Abcam), CD31 (R&D Systems), vascular endothelial (VE)-cadherin (R&D Systems), α-fetoprotein (AFP; R&D System), β-III-tubulin (Chemicon International, Inc., Temecula, CA, USA), or α-smooth muscle actin (α-SMA; R&D Systems), or with irrelevant non-specific IgGs. The cells were then incubated with Alexa Fluo 488- or 594-conjugated secondary IgGs (Invitrogen) and imaged using a fluorescence microscope (Nikon). Nuclei were stained with DAPI.
2.9 Small interfering RNA transfection

To silence the gene expression, hEBs were differentiated for 15 days and transfected with 50–200 nM of small interfering RNA (siRNA; Bioneer, Daejeon, Korea) using Lipofectamine™ 2000 (Invitrogen). Reverse transcription polymerase chain reaction (RT-PCR) was performed to analyse gene expression 24 h after transfection. The sequences of the siRNAs are listed in Supplementary material online.

2.10 Glutathione peroxidase assay

Cells were homogenized in cold buffer containing Tris–HCl (50 mM; pH 7.5), EDTA (5 mM), and dithiothreitol (1 mM). The cell homogenate was centrifuged and the supernatant was mixed with assay buffer (GPx Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) containing NADPH, glutathione, and glutathione reductase. Cumene hydroperoxide was added to initiate the reaction and the absorbance at 340 nm was measured every minute, with measurements obtained for at least five time points. Samples pretreated with MSA (100 μM) were included to measure non-specific NADPH oxidation. Glutathione peroxidase (GPx) activity was expressed as nanomoles of NADPH oxidized per minute and millilitre of sample.

2.11 Thioredoxin reductase assay

Cells were homogenized in cold buffer containing potassium phosphate (50 mM; pH 7.4) and EDTA (1 mM). The cell homogenate was centrifuged and the supernatant was mixed with assay buffer (TrxR Assay Kit; Cayman Chemical). NADPH and 5,5′-dithiobis-(2-nitrobenzoic acid) were added to initiate the reaction, and the absorbance at 405 nm was measured every minute, with measurements obtained for at least five time points. Samples pretreated with sodium aurothiomalate (20 μM) were included to measure non-specific thioredoxin reductase (TrxR) activity. TrxR activity was expressed as micromoles of NADPH oxidized per minute and millilitre of sample.

2.12 Statistical analysis

All data are presented as mean values ± standard errors of the means (SEM). One-way analysis of variance, followed by Bonferroni’s post hoc multiple comparison test, was used to determine the differences between multiple groups. Unpaired Student’s t-test was used for comparison between two groups. A P-value of < 0.05 was considered as statistically significant. The n number indicates the number of samples in each experiment.

3. Results

3.1 Quiescent hESC-derived CD34+ cells

To examine the expression levels of cell cycle-related genes in hESC-derived vascular progenitors, real-time RT-PCR analysis of hESC-derived CD34− cells and CD34+ cells containing vascular progenitors was performed. These cells were isolated using FACS from hESCs that had undergone vascular differentiation for 15 days (Figure 1A). A G1 checkpoint inhibitor (p21) that induces cell cycle arrest was markedly up-regulated, while several genes associated with cell cycle progression were down-regulated. In contrast, cell cycle-related genes in hESC-derived CD34− cells were expressed at higher levels than those in CD34+ cells.

Figure 1 hESC-derived CD34+ cells are quiescent. (A) Flow cytometric analysis of CD34+ cells in differentiated hEBs. (B) Real-time RT-PCR analysis of cell cycle-related genes in hESC-derived CD34+ and CD34− cells. CD34+ and CD34− cells were sorted from hEBs on Day 15 of vascular differentiation. mRNA levels in CD34− cells are expressed relative to the levels in CD34+ cells (set as 1) (mean ± SEM, *P < 0.05, n = 3). (C) Quantitative analysis of phospho-p53 in CD34− cells in hESC-derived CD34− and CD34+ cell populations. On Day 15 of vascular differentiation, sorted CD34− and CD34+ cells were plated at the same density and labelled with anti-phospho-p53 IgG. The mean number of phospho-p53+ cells per field is shown (mean ± SEM, *P < 0.05, n = 4). (D) Cell cycle analysis of hESC-derived CD34− and CD34+ cells. On Day 15 of vascular differentiation, CD34+ and CD34− cells were sorted with the same gating strategy used in (A). Sorted cells were then stained with pyronin Y and 7-AAD. Numbers indicate the percentage of cells in each cell cycle phase. Quiescent cells (G0 phase) were identified as the population with low 7-AAD and pyronin Y signals and quantified as the percentage of the total number of cells (mean ± SEM, *P < 0.05, n = 4). (E) Representative images of Ki-67-stained samples and the percentages of Ki-67+ cells in hESC-derived CD34− and CD34+ cell populations are shown (mean ± SEM, *P < 0.05, n = 4). On Day 15 of vascular differentiation, sorted cells were labelled with anti-Ki-67 IgG (green) and DAPI (blue). The percentage of Ki-67+ cells was expressed as the mean number of Ki-67+ cells per the total number of cells in a given image. Negative control (NC) samples were stained with isotype-matched control IgG. Scale bars indicate 50 μm.
(CCNB1, CDC20, CDK1, and PCNA) were down-regulated in hESC-derived CD34+ cells than in hESC-derived CD34- cells (Figure 1B). In addition, the number of phospho-p53+ cells was much greater in CD34+ than in CD34- cells. Consistent with this, cell cycle analysis showed that 41.0% of hESC-derived CD34+ cells were in G0 phase compared with 9.1% of hESC-derived CD34- cells (Figure 1D). Figure 1E and Supplementary material online, Figure S1 also showed that the percentage of Ki-67+ or pH3+ cells was significantly lower in the hESC-derived CD34+ cell population than in the hESC-derived CD34- cell population. Although Ki-67 and pH3 are not perfect markers of cell division or proliferation, overall results indicate that a large proportion of hESC-derived CD34+ cells are not actively dividing, remaining in a quiescent state during vascular differentiation of hESCs.

3.2 Generation of ROS in hESC-derived CD34+ cells

Cell cycle progression is controlled by cellular redox status. In particular, p21 has been demonstrated to be highly induced by ROS and controls cell cycle progression in a redox-sensitive manner. Thus, we hypothesized that ROS are associated with the quiescent state of hESC-derived CD34+ cells. First, we examined ROS generation during vascular differentiation of hESCs. The fluorescence intensity of H2DCFDA, an intracellular ROS indicator that becomes fluorescent upon oxidation by ROS, significantly increased during vascular differentiation of hESCs. The fluorescence intensity of H2DCFDA, an intracellular ROS indicator that becomes fluorescent upon oxidation by ROS, significantly increased during vascular differentiation of hESCs (Figure 2A). The cells with fluorescent H2DCFDA were mainly located at the centres of hEBs, the region in which CD34+ cells are primarily located. Immunofluorescence analysis of hEBs revealed that H2DCFDA fluorescence co-localized with PE-conjugated anti-CD34 IgG fluorescence (Figure 2B). In cells isolated from differentiated hEBs, the fluorescence intensity of H2DCFDA was significantly higher in CD34+ than in CD34- cells (Figure 2C and see Supplementary material online, Figure S2). This suggests that ROS are mainly produced in CD34+ cells during vascular differentiation of hESCs.

3.3 NADPH oxidase 4 generates ROS in hESC-derived CD34+ cells

ROS are generated from a number of sources, including the mitochondrial electron transport system, xanthine oxidase, NADPH oxidase (NOX), and uncoupled nitric oxide synthase (NOS). We examined the source of ROS produced during vascular differentiation of hESCs using pharmacological inhibitors that selectively block pathways of ROS generation. The dose of each inhibitor was optimized such that ROS generation was transiently inhibited without markedly affecting cell viability. Treatment with NAC, a flavoprotein inhibitor (DPI), or a NOX inhibitor (apocynin), a mitochondrial oxidative phosphorylation inhibitor (FCCP), or an endothelial NOS inhibitor (L-NAME) did not affect ROS generation in hESC-derived CD34+ cells (Figure 3A). However, treatment with a xanthine oxidase inhibitor (allopurinol), a mitochondrial oxidative phosphorylation inhibitor (FCCP), or an endothelial NOS inhibitor (L-NAME) did not affect ROS generation in hESC-derived CD34+ cells. These data suggest that NOX is a major source of ROS in hESC-derived CD34+ cells.

To further determine the isoforms of NOX involved in ROS generation, the expression profiles of five human NOX isoforms were analysed using RT-PCR (Figure 3B). The mRNA level of NOX4 initially decreased and then gradually increased from Day 12 of vascular differentiation, the point at which CD34+ cells began to appear in differentiated hEBs. The mRNA levels of NOX4 and p22phox were markedly higher in CD34+ than in CD34- cells (Figure 3C). Expression of the other NOX isoforms was not detected in CD34+ or CD34- cells. To investigate the involvement of NOX4 in ROS generation during vascular differentiation of hESCs, NOX4 expression was reduced in differentiated hEBs using siRNA (Figure 3D). In hEBs treated with NOX4-specific siRNA (Si-NOX4), few CD34+ cells displayed fluorescent H2DCFDA (Figure 3E). In contrast, in hEBs treated with control siRNA (Si-Cont), H2DCFDA fluorescence intensity was high in CD34+ cells. Quantitative analysis of H2DCFDA fluorescence revealed that ROS levels were significantly lower in Si-NOX4-treated hEBs than in Si-Cont-treated hEBs.

**Figure 2** ROS generation in hESC-derived CD34+ cells. (A) Representative images of H2DCFDA fluorescence in undifferentiated hESCs (D0) and hEBs cultured in vascular differentiation medium for 10 (D10) or 15 (D15) days, and quantitative evaluation of H2DCFDA fluorescence intensity (mean ± SEM, *P < 0.05 vs. D0, n = 4). Celluar ROS levels were measured using the redox-sensitive indicator H2DCFDA. Cells pretreated with 100 μM hydrogen peroxide were included as positive controls. As the NCs, cells were not incubated with H2DCFDA. (B) On Day 15 of vascular differentiation, hEBs were stained with PE-conjugated anti-CD34 IgG (red) and H2DCFDA (green). (C) Cellular ROS levels in hESC-derived CD34+ and CD34- cells. On Day 15 of vascular differentiation, sorted cells were stained with H2DCFDA and the fluorescence intensities of 30–50 cells in each group were quantified (mean ± SEM, *P < 0.05, n = 3). All scale bars indicate 100 μm.
3.4 Selenium reduces the levels of ROS in hESC-derived CD34⁺ cells by enhancing the antioxidant activities of TrxR and GPx

Next, we investigated whether the cell cycle status of hESC-derived CD34⁺ cells is altered when the level of intracellular ROS is reduced. Hydrogen peroxide, a major ROS produced by NOX4, is neutralized into water by catalase and the glutathione and thioredoxin redox systems. To reduce intracellular ROS levels, NAC, which completely scavenges ROS similar to catalase, was added to the differentiation culture medium of cells. NAC treatment almost completely inhibited vascular differentiation of hESCs (see Supplementary material online, Figure S3), suggesting that a certain level of ROS is required for vascular differentiation. To moderately reduce the level of ROS, the antioxidant activities of the glutathione and thioredoxin systems were modulated using selenium. TrxR and GPx are key enzymes in these systems and both are selenoproteins that require selenium for their activities. The mRNA levels of TrxR and GPx in hESC-derived CD34⁺ cells were examined (Figure 4A). All TrxR genes (TrxR1, TrxR2, and TrxR3) and four GPx genes (GPx1, GPx2, GPx3, and GPx4) were expressed in hESC-derived CD34⁺ cells. When hEBs were differentiated in the presence of non-cytotoxic concentrations of selenium (20 and 50 ng/mL), the antioxidant activities of TrxR and GPx were enhanced and the levels of ROS in hEBS were substantially reduced by Day 15 of differentiation (Figure 4B–D). The fluorescence intensity of H2DCFDA was significantly lower in selenium-treated hESC-derived CD34⁺ cells than in untreated hESC-derived CD34⁺ cells. To confirm that changes in ROS levels in hESC-derived CD34⁺ cells in response to selenium treatment were due to the enhanced activities of TrxR and GPx, levels of ROS were measured after treatment with a TrxR inhibitor (ATG, AF, or PX12) or a GPx inhibitor (MSA). Treatment with each of these inhibitors substantially increased the fluorescence intensity of H2DCFDA in selenium-treated CD34⁺ cells, suggesting that selenium reduces ROS levels in hESC-derived CD34⁺ cells by enhancing the antioxidant activities of TrxR and GPx (Figure 4F).
3.5 Selenium enhances the proliferation of hESC-derived CD34+ cells

Next, we determined whether the decrease in ROS levels following selenium supplementation changes the cell cycle status of hESC-derived CD34+ cells. There were significantly more Ki-67+ cells in the selenium-treated hESC-derived CD34+ cell population than in the untreated hESC-derived CD34+ cell population (Figure 5A). Cell cycle analysis showed that the percentage of cells in G0 phase was lower in the selenium-treated hESC-derived CD34+ cell population than in the untreated hESC-derived CD34+ cell population (Figure 5B). In RT-PCR analysis, the mRNA levels of mitosis-related genes (CDK1, CCNA2, CCND1, CCNB1, CDC20, and PCNA) were increased in selenium-treated CD34+ cells, whereas expression of p21 was substantially reduced (Figure 5C). Flow cytometric analysis showed that selenium treatment increased the number of CD34+ cells during vascular differentiation of hESCs (Figure 5D). This result was confirmed by immunocytochemical data, showing that the number of CD34+ cells was substantially increased in selenium-treated hEBs (Figure 5E).

3.6 Selenium promotes vascular differentiation of hESCs

Since CD34+ cells differentiate into ECs and SMCs during the differentiation of hESCs, we examined whether the selenium-induced increase in the number of CD34+ cells enhances vascular differentiation of CD34+ cells, whereas expression of p21 was substantially reduced (Figure 5G). Flow cytometric analysis showed that selenium treatment increased the number of CD34+ cells during vascular differentiation of hESCs (Figure 5D). This result was confirmed by immunocytochemical data, showing that the number of CD34+ cells was substantially increased in selenium-treated hEBs (Figure 5E).
hESCs. Real-time RT-PCR showed that mRNA levels of EC-specific genes (CD31 and VE-cadherin) and SMC-specific genes (Myocardin and α-SMA) were higher in selenium-treated hEBs than in untreated hEBs (Figure 6A). Flow cytometric analysis showed that there were more CD31+ cells and smooth muscle22α (SM22α)+ cells in selenium-treated hEBs than in untreated hEBs (Figure 6B). Consistent with the RT-PCR results, immunocytochemical data revealed that protein levels of CD31, VE-cadherin, and α-SMA were substantially higher in selenium-treated hEBs than in untreated hEBs (Figure 6C). The selenium-induced increase in vascular differentiation of hESCs was abrogated when hEBs were co-treated with selenium and a TrxR inhibitor (ATG) or a GPx inhibitor (MSA) (see Supplementary material online, Figure S4). This suggests that selenium promotes vascular differentiation of hESCs by enhancing the activities of TrxR and GPx. In contrast, selenium supplementation did not affect the differentiation of hESCs into endoderm or ectoderm (see Supplementary material online, Figure S5).

4. Discussion

In vascular biology, ROS are critical regulators of diverse physiological and pathophysiological processes. However, the specific roles of ROS during human embryonic vascular development have not been fully investigated. In this study, hESCs were used to investigate the generation, source, and role of ROS during vascular differentiation. NOX4 expression was substantially higher in CD34+ than in CD34- cells. NOX4 constitutively generates ROS (mainly hydrogen peroxide) without the need for a stimulus, and the level of NOX4-induced ROS production is mostly determined by the NOX4 expression level. Indeed, knockdown of NOX4 using siRNA significantly reduced the level of ROS in CD34+ cells, indicating that NOX4 is the predominant source of ROS in hESC-derived CD34+ cells. NOX4 expression is induced in response to a wide variety of agonists and cellular stressors. In particular, several NOX4 inducers, such as BMP4 and angiotensin II, as well as hypoxia, are crucial mediators of the vascular differentiation of hESCs. BMP4 promotes the development of vascular progenitors during the differentiation of hESCs. Zambidis et al. showed that inhibitors of angiotensin II signalling significantly reduce the expansion of hESC-derived haemangioblasts that give rise to vascular progenitors and haematopoietic stem cells. Hypoxia has recently been shown to accelerate the differentiation of murine ESCs into vascular lineage cells. Our previous study revealed that CD34+ cells appear mainly at the centre of hEBs, which might be subjected to severe hypoxia relative to the periphery of hEBs. Given the importance and relevance of NOX4 inducers in the vascular differentiation of hESCs, BMP4, angiotensin II, and/or hypoxia may be involved in the up-regulation of NOX4 expression in hESC-derived CD34+ cells.
The present study implicated NOX4-induced ROS generation in the quiescence of hESC-derived CD34⁺ cells. Unlike CD34⁻ cells, CD34⁺ cells exhibited a gene expression pattern that is characteristic of non-dividing cells. For example, genes involved in mitosis (CCNB1, CCDC20, and CCND1) and DNA replication (PCNA) were significantly down-regulated in CD34⁺ cells. In particular, the number of phospho-p53⁺ cells in the hESC-derived CD34⁺ cell population is substantially greater than in the hESC-derived CD34⁻ cell population. This suggests that p53 pathway may be highly activated in hESC-derived CD34⁺ cells. Given that p53 directly increases the transcription of p21, a significant increase in the mRNA level of p21 in hESC-derived CD34⁺ cells may be due to p53 activation. p21 is a molecular switch governing transition out of the cell cycle and maintenance in G₀ phase. The expression of p21 is regulated in a redox-sensitive manner. In various cell types, an increase in endogenous ROS or treatment with sublethal doses of hydrogen peroxide induces cell cycle arrest that is associated with increased expression of p21. In the present study, the mRNA level of p21 in hESC-derived CD34⁺ cells was substantially reduced by selenium treatment that moderately reduced ROS levels by enhancing the antioxidant activities of TrxR and GPx. In addition, the expression levels of other genes involved in cell proliferation (CCDK1, CCNA2, CCNB1, CCND1, CDC20, and PCNA) were increased in selenium-treated CD34⁺ cells, which led to a decrease in the percentage of cells in G₀ phase and an enhanced proliferation rate. These data suggest that NOX4-induced ROS generation may be closely associated with the quiescent state of CD34⁺ cells during vascular differentiation of hESCs. It was further supported by the siRNA-mediated knockdown results that a moderate knockdown of NOX4 or p22⁹⁰⁻⁻ decreased ROS levels and subsequently enhanced the proliferation and vascular differentiation of hESC-derived CD34⁺ cells (see Supplementary material online, Figures 6 and 7).

Although a moderate reduction in the level of ROS using selenium caused quiescent CD34⁺ cells to enter the cell cycle and proliferate, complete scavenging of ROS using NAC inhibited vascular differentiation of hESCs. During angiogenesis, NOX-dependent ROS formation is required to enhance EC proliferation and migration. In ischaemic vascular disease models, administration of NAC and NOX inhibitors abrogates collateral vessel growth and neovascularization in injured tissues. NOX-derived ROS inactivate protein tyrosine phosphatase and therefore enhance signalling cascades for angiogenic factors, such as VEGF, bFGF, stromal derived factor-1, and erythropoietin. Furthermore, NOX4-derived hydrogen peroxide increases the expression and phosphorylation of endothelial NOS. A moderate level of NOX4-derived ROS may exert similar effects in the regulation of signalling pathways and gene expression required for vascular differentiation of hESCs. However, more research is needed to elucidate the molecular mechanisms through which ROS regulate the vascular differentiation of hESCs.

Taken together, this study demonstrates that NOX4-derived ROS are produced in CD34⁺ cells during the vascular differentiation of hESCs and are implicated in the quiescent state of hESC-derived CD34⁺ cells. A moderate decrease in ROS levels using selenium significantly promotes the proliferation of CD34⁺ cells, thereby enhancing the vascular differentiation of hESCs. However, it cannot be ruled out that other mechanisms might be involved in the cell cycle control of hESC-derived CD34⁺ cells. These findings may contribute to a better understanding of redox states in human embryo during vascular differentiation and help to optimize the conditions required for efficient vascular differentiation of hESCs.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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