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RESEARCH ARTICLE

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Comparing the *in vivo* and *in vitro* effects of hypoxia (3% O_2) on directly derived cells from murine cardiac explants versus murine cardiosphere derived cells

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Abstract

Coronary heart disease (CHD) is still one of the main causes of death in the world, despite significant advances in clinical treatments. Stem cell transplantation methods have the potential to improve cardiac function and patients' outcome following heart attack, but optimal cell types, cell preparation methods and cell delivery routes are yet to be developed. Mammalian hearts contain a small fraction of progenitor cells which, in culture, migrate out of the cardiac explants, known as explant-derived cell (EDCs) and contribute to spheroids known as cardiospheres (Csphs). Following further culture and cell passaging, Csphs give rise to cardiosphere-derived cells (CDCs). EDCs, Csphs and CDCs show *in vitro* and *in vivo* angiogenesis and tissue regeneration in myocardial ischemia. However, CDC and Csph formation is time consuming, expensive and not always successful. Therefore, this study aims to compare EDCs with CDCs and assess the effect of hypoxic preconditioning on their pro-angiogenic potential. The data showed that preconditioning EDCs in hypoxic cell culture enhances cell growth, viability and expression of stem cell and pro-angiogenic markers more than CDCs. *In vivo* experiments using a sub-dermal matrigel plug assay showed that EDCs and CDCs alone have limited pro-angiogenic potential; however, hypoxic preconditioning of EDCs and CDCs significantly enhances this process. Further research will increase our understanding of cardiac stem cell mediated angiogenesis and improve clinical therapies for myocardial infarction (MI) patients.

Key Words: Coronary heart disease (CHD); Stem cell transplantation, Cardiac progenitors; Explant-derived cell (EDCs); Cardiospheres (Csphs), Cardiosphere-derived cells (CDCs); Hypoxia; Angiogenesis.

Introduction

Stem cell transplantation studies following heart attack aim to establish a way to supply adequate blood and reduce the infarct size. Transplantation of cardiac progenitor cells (cardiosphere-derived cells (CDCs) and cardiospheres (Csphs)) has been shown to be effective either by paracrine or endocrine neo-vascularisation[1-4]. However, a major dilemma in stem cell transplantation studies is their low survival rate after transplantation. Therefore, improving cell retention after transplantation such as hypoxia preconditioning of the candidate cells could improve the efficiency of stem cell therapy. Hypoxia preconditioning has been shown to stimulate numerous endogenous mechanisms such as reducing apoptosis and enhancing myocyte protection[5]. Studies have shown that hypoxia preconditioning of EDCs and CDCs markedly improves cell migration (*in vitro*) and cell recruitment into the ischemic myocardium[6-10]. Here, we showed that the expression of stem cell (Sca-1, Abcg2), endothelial and angiogenic markers (Eng, Flk1, Vegf) increased significantly in EDCs and CDCs preconditioned with hypoxia and interestingly, this response was more significant in EDCs. In order to assess the ability of CDCs and EDCs to promote neovascularization *in vivo*, we used a mouse model of angiogenesis with subcutaneous injection of matrigel combined with CDCs or EDCs. The results showed that EDCs and CDCs have limited angiogenic capacity alone. However, this characteristic was significantly enhanced in both cell types upon preconditioning with hypoxia (3% O_2).

Material and methods

Mice

Mice (C57BL/6) were obtained from Jackson laboratory and CAG-farnesylated-eGFP mice were obtained from Prof. A. Medvinski (Edinburgh University). All animal experiments were performed under a UK Home Office Licence and the Iranian National Institute of Genetic Engineering and Biotechnology (NIGEB) guidelines for laboratory animal care.

Cell culture

Adult murine cardiac stem cells were expanded using a similar method as described by Amirrasouli[11]. Briefly, following dissection of adult murine hearts, they were minced into small fragments and digested with 0.05% trypsin for 10 minutes. The partially digested tissue fragments were then moved to 35 mm cell culture plates pre-coated with 1 mg/ml fibronectin (BD Biosciences)

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and cultured in IMDM (Gibco Invitrogen) supplemented with 20% foetal calf or bovine serum (heat inactivated), 1% L-Glutamine (Gibco Invitrogen), 0.1mM 2-mercaptoethanol (Gibco Invitrogen), 100 unit/ml penicillin and 100µg/ml streptomycin. After 4 weeks, the explant cultures were randomly selected to culture in a typical CO_2 incubator normoxia (20% O_2, 48 hours) or hypoxia (3% O_2 /48 hours).

Csphs and CDC culture

To culture Csphs, EDCs were seeded at a density of 10^5 cells per well of a 24-well plate pre-coated with 1.2 µg/ml polyD lysine (BD Biosciences) with cardiosphere growth medium containing: DMEM F12 (Gibco Invitrogen) supplemented with 2% B27 (Gibco Invitrogen), 80 ng/ml bFGF, 20 ng/ml EGF, 40nmol/L Cardiotropin - 1, 40nmol/L thrombin and 0.1 mmol/L (all from PeproTech) and 2-mercaptoethanol (Gibco Invitrogen). At days 12-14, loosely adherent Csphs were gently collected from the cell culture supernatant and cultured as CDCs. Finally, CDCs were grown from Csphs and expanded until passage 2 (P2). The CDC culture media was the same as for EDCs.

Quantitative PCR

Murine primer sequences were obtained from the PrimerBank website. In order to eliminate any genomic contamination, a BLAST search was carried out in order to confirm the specificity and coverage of exon boundary regions for each primer pair (Table 1). Total RNA was isolated from approximately 10^6 cells using a Qiagen RNA isolation kit following the manufacturer's instructions. cDNA preparation was performed using cDNA reverse transcription kit (Applied Bioscience) from 1µg RNA. Real-time PCR experiments were carried out by SYBR Green Master Mix (Qiagen) and the thermal profile for all qRT-PCR reactions was 95°C for 10 min, followed by 40 cycles of 95°C for 10s and 60°C for 30s using an ABI Fast Real Time PCR System (7900 HT). All samples were analysed in triplicate with three house keeping genes used as internal controls (Gapdh, β-actin and Rps19).

Table1.The list of primers used for qRT-PCR analysis. All primers are from the Primer Bank available at pga.mgh.harvard.edu

Gene	Sequence)5'-3')	Size (bps)	Tm (°C)
Gapdh	F: AACTTTGGCATTGTGGAAGG R: AGAACATCATCCCTGCATCC	132	60
β-actin	F: GGCTGTATTCCCCTCCATCG R: ACATGGCATTGTTACCAACTGG	154	60
Rps-19	F: GCTTGCCTCTAGTGTCC R:TGAGACCAATGAAATCGCCAA	75	60
Sca-1	F: TCAGGAGGCAGCAGTTATTGTG R: CGTGAAGACTTCCTGTTGCCA	160	60
cKit	F: CTCCCCCAACAGTGTATTCAC R: TAGCCCGAAATCGCAAATCTT	90	60
Abcg2	F: GATGAACTCCAGAGCCGTTAGGAC R:AACCTGGCCTTAATGCTATTCTG	169	60
Eng	F: CTGCCAATGCTGTGCGTGAA R: ACTTGGCCTACGACTCCAGCC	191	60
Vegf	F: CTTGTTCAGAGCGGAGAAAGC R: AACGAACGTACTTGCAGAGTG	125	60
Stat3	F: CACCTTGGATTGAGAGTCAAGAC R:AGGAATCGGCTATATTGCTGGT	112	60
Bcl-x	F:TTCGGGATGGAGTAAACTGGG R:CTCCTTGTCTACGCTTTCCAC	77	60

Western blotting

CDCs or EDCs were treated in normoxia or hypoxia for 48 hours before preparing whole cell protein lysates in SDS buffer. Proteins were separated on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride membrane. Then the samples were blocked with 5% powdered milk/TBST and incubated with primary antibody (HIF-1α, Santa Cruz) or β-actin (Sigma). Membranes were incubated with secondary antibody either anti-rabbit HRP in blocking solution for densitometric analysis all samples were assessed ImageQuant TL v2005 software

Immunofluorescence staining and fluorescent activated cell sorting (FACS)

For fluorescent cell staining, cells were blocked with blocking solution (5% goat serum, 1% BSA and 0.5% Tween 20). Then the appropriate primary antibody (Table 2) was added and incubated at 4°C overnight. Following cell wash, the matching secondary antibody (Table 2) was applied and finally mounted with hard set mountant (Vector Laboratories) containing DAPI. For visualisation, ZeissAxio Imager II with apotome was used. For FACS analysis, 10^5 cells in 100µl volume were incubated with an appropriate primary antibody (Table 2). Following the incubation time and cell wash, all tubes were incubated with the appropriate secondary antibodies. Finally, the cells were washed and DAPI (1µg/ml) was added. FACS analysis was performed with LSRII or FACS Canto (BD Biosciences) and the data was analysed by FACSDiVa software. Each analysis was performed with 10^4 events and all viable cells were included in the final data analysis. DAPI staining was used to provide a live/dead cell gate. Separate isotype and negative controls were used in all markers.

Table 2. The list of antibodies used for Immunophenotyping, Immunostaining of EDCs or CDCs and immunohistochemistry staining.

Antibody	Source and catalogue number	Working concentration
Sca-1	Ebioscience (17598181)	1/100
cKit	Ebioscience (171171)	1/100
CD90	BD Pharmingen (105201)	1/100
CD31	BD Pharmingen (102413)	1/100
CD34	BD Pharmingen (128605)	1/100
CD45	BD Pharmingen (103121)	1/100
Flk1	Ebioscience (565821)	1/100
Eng	Ebioscience (171057)	1/100
HIF1-α	Santa Cruz (sc-10790)	1/50
Alexa 594	Invitrogen (A11012)	1/200
Alexa 488 (anti GFP)	Invitrogen (A21311)	1/50
Alexa 647	Invitrogen (A-21247)	1/100
α-SMA Cy3.3	Sigma (C6198)	1/200

Side population studies

EDCs or CDCs were resuspended at the density of 10^6 cells/ml in PBS with 5% FBS. The cells were incubated in 1 µg/ml Hoechst 33342 dye for 60 min at 37°C (light protected), with or without 50 µM verapamil (Sigma). After the incubation time, cells were analysed for Hoechst 33342 (Sigma) dye efflux by LSRII flow cytometric analysis in hypoxic and normoxic samples. Using a UV laser, Hoechst 33342 dye was excited at 350 nm and fluorescent emission was detected through 450nm (Hoechst blue) and 675nm (Hoechst red) filters, respectively.

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Enzyme-Linked Immunosorbent Assay (ELISA)

10^5 EDCs or CDCs were seeded per well of a 12-well plate and 1.5 ml of media was added into each well. Cells were incubated in normoxia or hypoxia. At approximately 90% cell confluency, the cell culture medium was removed and centrifuged. VEGF levels in the cell supernatant were measured with a VEGF ELISA kit following the manufacturer's instructions (R & D systems) and the optical density was measured with Stat Fax 2100(Awareness Technology Inc.) at 560 nm.

In vivo angiogenesis

Growth factor reduced Matrigel (BD Biosciences) was thawed at 4°C and kept on ice. Anaesthesia was induced using an anaesthetic chamber (97% O_2/2% isoflurane) for 3 minutes. The flanks were shaved and cleaned with 70% ethanol. EDCs or CDCs derived from CAG-farnesylated-eGFP or C57BL/6 strains were suspended in 600µl matrigel and injected sub-cutaneously into the flank regions. After 10 days, the animals were humanely killed and matrigel plugs were dissected. Plugs were washed and fixed with 0.2% PFA at 4°C/overnight and then incubated with 30% sucrose at 4°C/overnight. Finally, the plugs were frozen by OCT freezing medium and sectioned using Microm HM 560 Cryostat (Thermo Scientific) and the sections were mounted on slides. For micro vessel density (MVD) analysis, matrigel plugs were stained with CD31 antibody as described previously[11]. Briefly, matrigel plug slides were air-dried, washed with PBS and blocked with a blocking solution (5% rabbit serum). The complementary blocking was carried out with Avidin/Biotin buffers (Vector Laboratories) to block unspecific sites. All sections were then incubated with a primary anti-CD31 antibody (BD Pharmingen) at 4°C/overnight. The next day, the slides were washed and incubated with secondary rabbit anti-rat antibody (Vector Laboratories) for 30 minutes at room temperature. All sections were washed again and incubated with ABC reagent for 30 minutes. After washing with PBS, all sections were incubated for 1 minute with the liquid DAB kit (BioGenex) and mounted with histomount (National Diagnostics) following ethanol dehydration. MVD was measured using ImageJ software and the intensity of the staining was normalised against the entire surface area of the matrigel and compared between the groups.

Microscopy

Light microscopy was used to assess cell morphology and growth using a Zeiss Axiovert 200 inverted microscope. For fluorescent-stained sections and Histochemical stained sections, a LeitzDiaplan Fluorescent microscope was used. All images were taken by digital camera and analysed with Image J to measure the staining intensity.

Statistical analysis

SPSS software (version 19, SPSS Inc.) was used to analyse the data. To test the normality, the Shapiro-Wilk test was used and multiple groups of data were analysed by one-way ANOVA followed by Post-Hoc (Tukey test). In all experiments, the paired t-test and p-value of <0.05 were defined as being statistically significant.

Results

Cardiac progenitor cell culture and HIF-1α stabilisation in hypoxic culture of EDCs and CDCs

Cardiac progenitors were obtained from adults C57BL/6 mice hearts and cultured in normoxia or hypoxia as described in (Amirrasouli, 2014)[11]. Figure 1 schematically represents different stages of EDC, Csph and CDC culture.

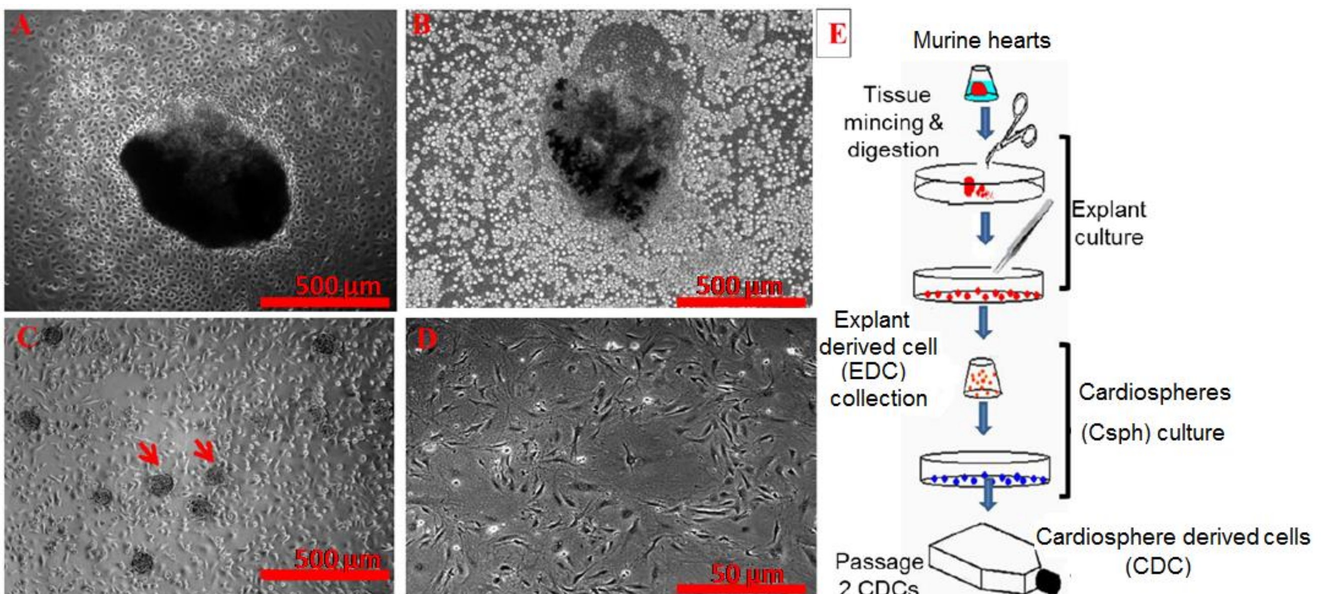

Figure 1. Different stages of cardiac progenitor cell culture. (A) Murine cardiac explant at day 6 of EDCs culture. (B) The same explant at day 14 with migrated phase bright cells and underlying stromal cells (C) Csphs at day 6 (arrows). (D) P2 CDCs, the scale bar is 500µm (A-C) and 50 µm in D. (E) Schematic representation of EDC, Csph and CDC culture. Modified from Amirrasouli 2014[11].

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To validate the hypoxic environment, HIF-1α stabilisation in EDCs and CDCs was evaluated by western blot and immunocytochemistry. EDCs and P2 CDCs cultured in normoxia or hypoxia, were analysed based on the stabilisation of the HIF-1α protein (Figure 2). Detailed HIF-1α protein quantification showed that EDCs stabilised HIF-1α twice as much as CDCs upon hypoxic culture induction.

Hypoxia increased the expression of anti-apoptotic markers in EDCs better than CDCs

As the role of Stat3/ Bclx pathway is shown to down regulate apoptosis *in vitro*[12],propagation of EDCs and CDCs under hypoxia showed a significant increase of Stat3/Bclx expression in comparison to normoxia control group (EDCs; stat3: 5.2 fold and Bclx: 3.9 fold, $p<0.05$), (CDCs; stat3: 2.1 fold and Bclx: 3.7 fold, $p<0.05$)(Figure 3). As EDC culture could take one month, the expression of stat3 was analysed with qRT-PCR at four time points (end of weeks 1 to 4) (Figure 3C). As shown in Figure 3, the mean mRNA level of stat3 marker in EDCs remained constant in hypoxic environment.

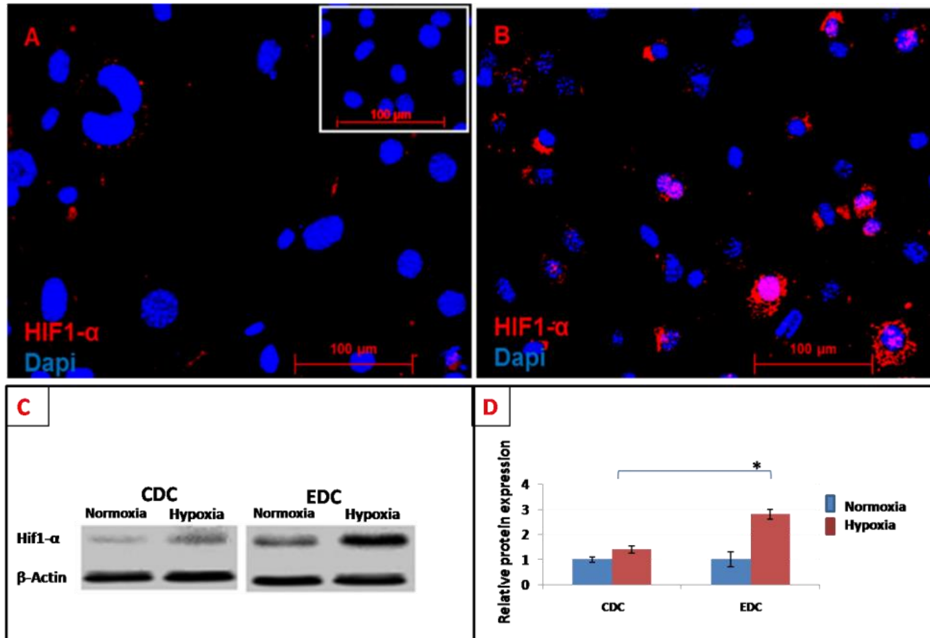

Figure 2. HIF-1α stabilisation in EDCs and P2 CDCs cultured in normoxia or 3%O_2. CDCs were stained with anti-HIF-1α specific antibody and detected with an anti-rabbit secondary antibody conjugated with alexa 594 (red). CDCs were cultured in (A) normoxia or (B) hypoxia for 48 hours and DAPI (blue) was used to stain nuclei, 2A and 2B are from[11]. (C) Representative western blot from EDCs or CDCs cultured in normoxia or hypoxia to analyse HIF-1α stabilisation. D) The summary of densitometric analysis of HIF 1-α band intensity in CDCs and EDCs cultured in hypoxia and normoxia relative to β-actin are shown *$p<0.05$. Scale bar = 100 µm. The inset image in (A) is the negative control (no primary antibody) with the same magnification. Figures (A) and (B) are modified from Amirrasouli 2014[11].

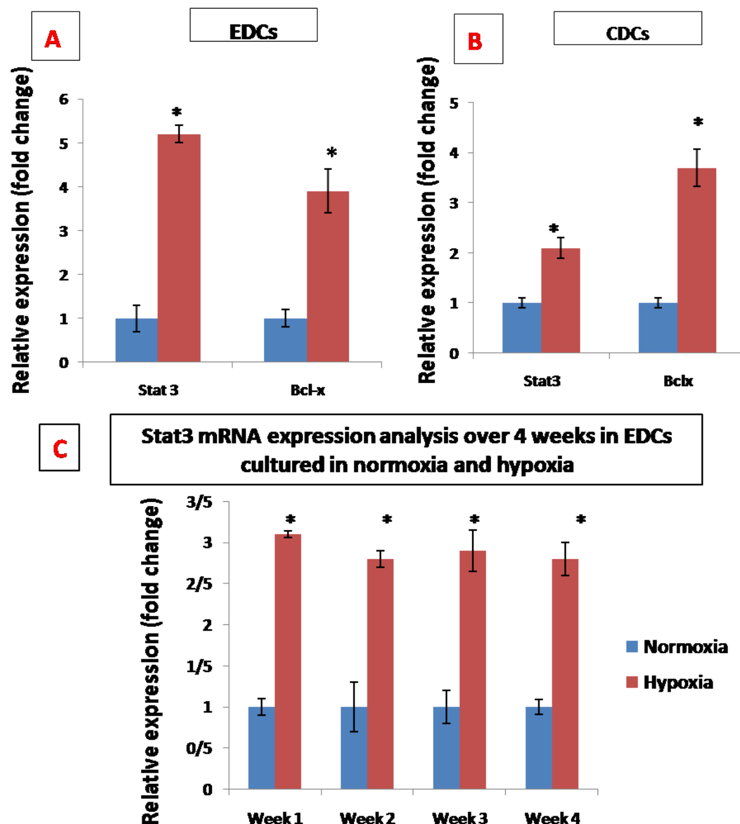

Figure 3. The effect of hypoxia on mRNA expression of anti-apoptotic markers (stat3-Bclx) on EDCs (A) and CDCs (B) compared to normoxia control groups. (C) The analysis of stat3 mRNA level in EDCs cultured in hypoxia 3% O_2 and normoxia for 4 weeks.* $P<0.05$

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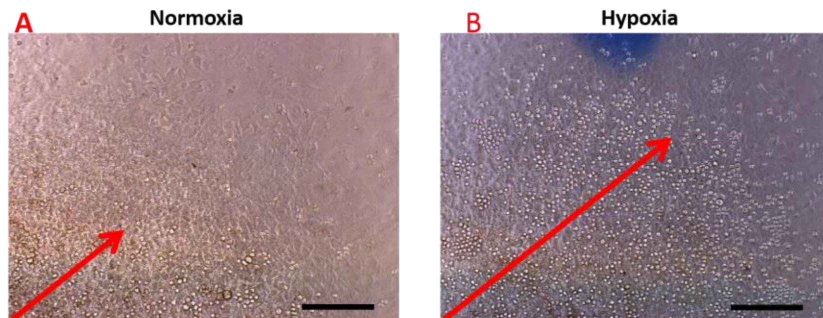
The effect of hypoxia on EDCs' outgrowth from cardiac explants

It is already shown that hypoxia stimulates cell migration through the perk/atf4/lamp3 pathway[16]. Therefore, to validate the effect of hypoxia on EDCs' migration, we cultured murine cardiac explants from C57BL/6 strain in hypoxia and normoxia for two weeks. The length of cell outgrowth was measured at several points from each explant. The results showed the positive effect of hypoxia on increasing EDCs' outgrowth (1236±48.3 µm in normoxia vs. 2420.7±124µm in hypoxia, $p<0.05$). However, our initial studies in Newcastle University on Csphs and CDC proliferation in hypoxia did not show any significant difference compared to normoxia counterparts[11]. Figure 4 shows the effect of hypoxia treatment on EDC cultures.

Hypoxia increases the expression of cardiac stem cell and angiogenic markers in EDCs more than CDCs

Using qRT-PCR and FACS, the level of cardiac stem cell (Sca-1, cKit, Abcg2), endothelial (CD31 and CD34) and angiogenic cell markers (VEGF, Eng & Flk1) were analysed in EDCs and CDCs treated with hypoxia or normoxia. At mRNA level (Figure 5A-B), Sca-1 showed the highest expression among stem cell markers in EDCs (6.2-fold, Figure 5A, $p<0.05$) and CDCs (2.7-fold, figure 5B, $p<0.05$). Although, cKit mRNA remained unchanged in CDCs (1.3-fold, Figure 5B, p=ns), it showed a significant up-regulation in hypoxia EDCs (3.1-fold, Figure 5A, $p=0.05$). Hypoxia also increased the level of angiogenic markers (Eng, VEGF) and these enhancements were more evident in EDCs (VEGF: 4.2-fold and Eng: 4.3 fold $p<0.05$) (Figures 5A and 5B).



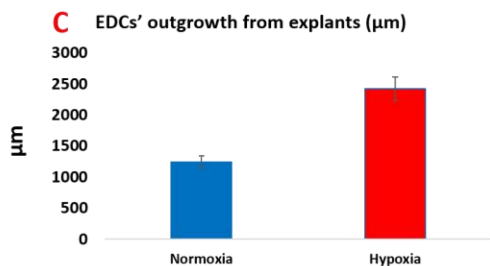


Figure 4. **The effect of hypoxic cell culture on EDCs' outgrowth**. Normoxia (A) and (B) hypoxia. Red arrows are indicating the outgrowth length from the explant. (C) The summary of EDCs' outgrowth compared in normoxia and hypoxia. *$p<0.05$ Scale bar = 500µm.

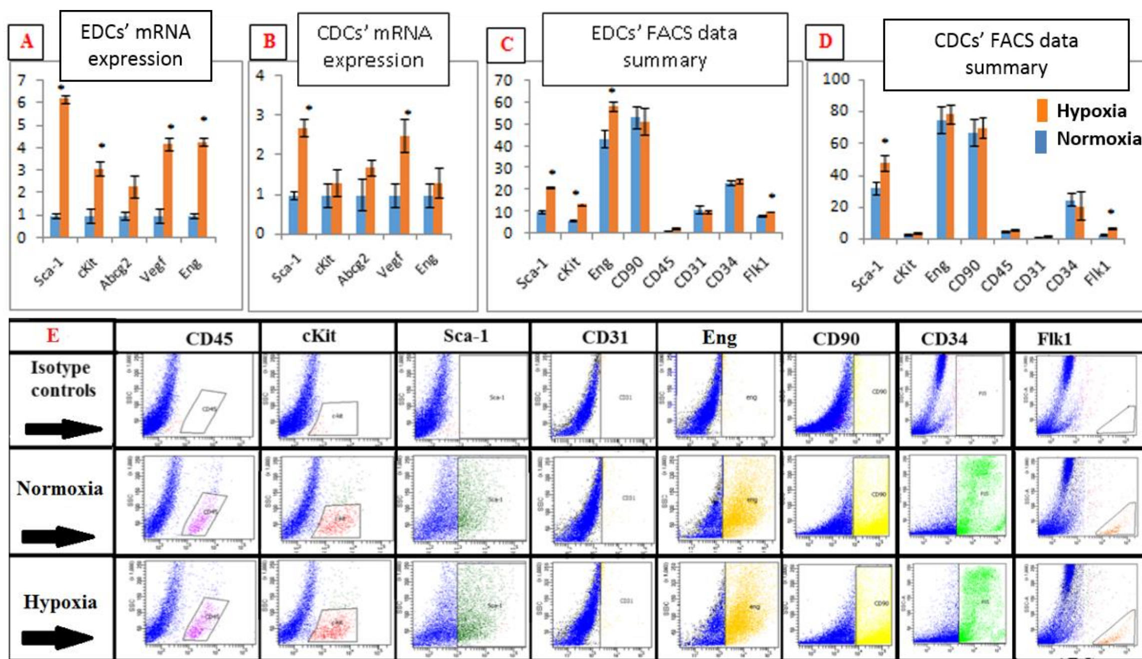


Figure 5. **The effect of hypoxia on EDC and CDC mRNA and cell surface markers**. The comparison of the effect of hypoxia on EDCs mRNA (A), CDCs mRNA (B) cultured in normoxia or hypoxia. Using qRT-PCR the level of stem cell (Sca-1, cKit and Abcg2), angiogenic and endothelial (Vegf and Eng) markers were evaluated in EDCs or CDCs. Protein expression of stem cell (Sca-1, cKit,) Mesenchymal (CD90-Eng), Hematopoietic (CD45), endothelial and angiogenic (CD31, CD34 and Flk1) markers were assessed in EDCs (C) and CDCs (D). Blue and orange bars (A-D) are representing normoxic and hypoxic cell cultures in both qRT-PCR and FACS data respectively. (E) Representative FACS plots form CDC culture, modified from[11].*$p<0.05$.

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To further evaluate the increase seen in gene expression of EDCs and CDCs, we used flowcytometry in order to validate the results obtained by qRT-PCR. Therefore, the panel of endothelial (Flk1, CD34, CD31), mesenchymal (Eng, CD90), haematopoietic (CD45) and stem cell (Sca-1, cKit) markers were used. Figure 5E shows a representative FACS plots from CDC culture and 5C & D summarise the results as a percentage of positive events for all cell populations analysed in EDCs and CDCs respectively. Although, CDCs cultured in hypoxia showed higher levels of cKit, Eng and CD34, only the level of Sca-1 (32±4 vs 48±5, Figure 5D, $p<0.05$) and Flk1 (3±0.1 vs 6.8±0.3, figure 5D, $p<0.05$) reached to significant levels between two groups. Interestingly, in hypoxia EDCs, Sca-1 (10±1 vs 21±0.5, figure 5C, $p<0.05$) and Flk1 (8±0.2 vs 10.1± 0.1, Figure 5C, $p<0.05$) levels were significantly higher than their normoxia control groups. The difference in cKit (6±0.5 vs 13.4±0.8, $p<0.05$) and Eng (43.5±4 vs 58±2, $p<0.05$) protein expression were also higher in hypoxia EDCs (Figure 5C). More importantly the data showed that a very low percentage of EDCs or CDCs expressed CD45. Therefore, it is not likely that these cells were originated from hematopoetic tissues. Figure 5 summerises the data from qRT-PCR and FACS experiments, (blue and orange bars are representing normoxic and hypoxic cell culture conditions respectively).

Hypoxia increases the secretion of VEGF in EDCs' supernatant more than in CDCs'

To confirm the up-regulation of VEGF mRNA expression following hypoxia culture, we analysed the level of VEGF in the supernatant of EDCs' and CDCs' with the same cellular density cultured in normoxia or hypoxia. Using ELISA, we observed that hypoxia significantly increased VEGF protein in the supernatant of EDCs' (210±25µg/ml vs 520± 17µg/ml, $p<0.001$) and CDCs' (128±19µg/ml vs 223 ± 26µg/ml, $p<0.001$) in comparison with the normoxia counterparts. Interestingly, the level of VEGF was more evident in the supernatant of hypoxia EDCs' (Figure 6).

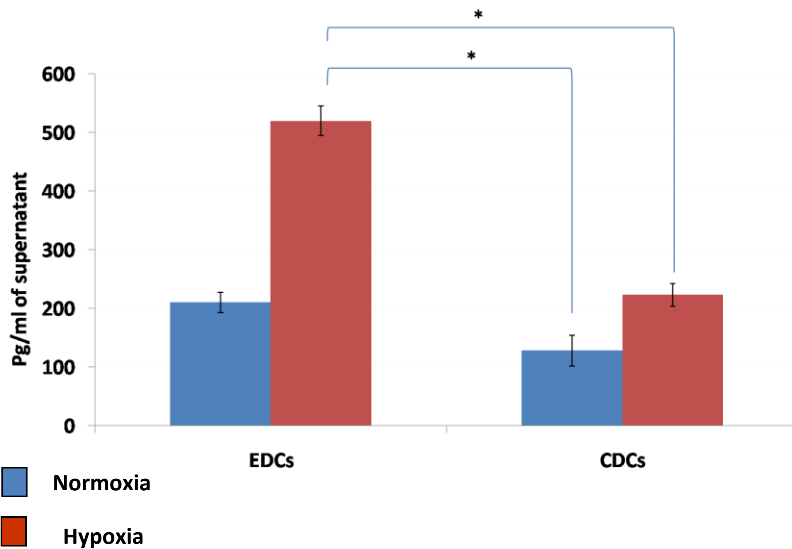

Figure 6.The level of VEGF in the supernatant of EDCs and CDCs cultured in normoxia or hypoxia. The VEGF level was measured with a specific murine VEGF ELISA kit.* $p<0.001$ Blue and red bars are representing normoxic and hypoxic cell culture conditions.

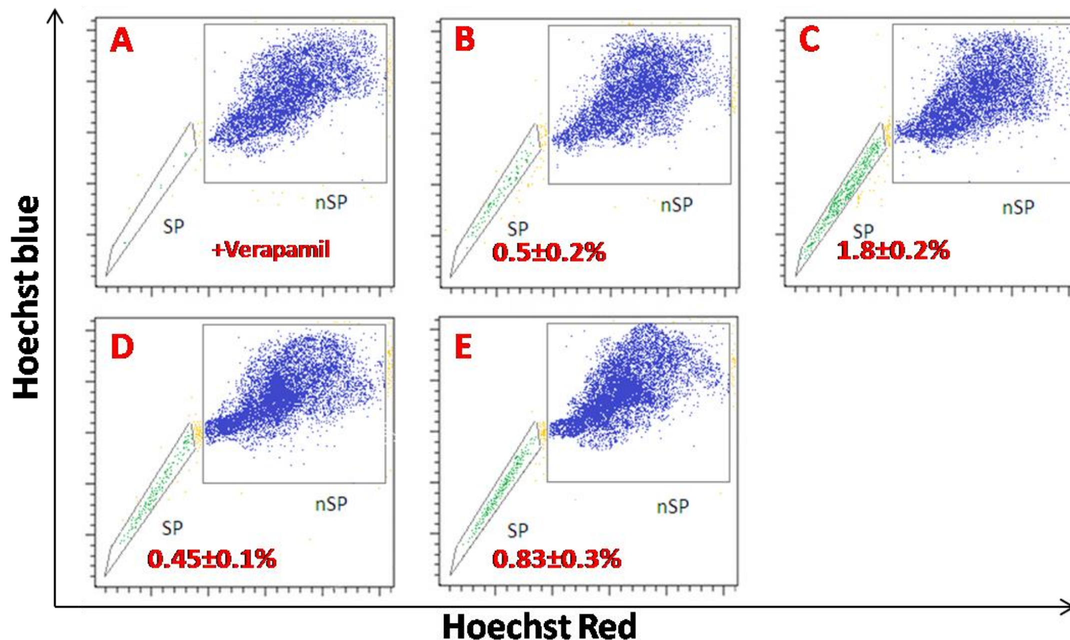

Figure 7: **The effect of hypoxia on EDC and CDC, side population percentage**. (A) EDCs with Verapamil, (B) EDCs in normoxia, (C) EDCs in hypoxia, (D) CDCs in normoxia and (E) CDCs in hypoxia. $p<0.05$

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The effect of hypoxia on side population cells of EDCs and CDCs

It has been shown that cardiac side population cells contain putative cells with the ability to pump out the dye Hoechst 33342[13]. EDCs or CDCs cultured in hypoxia or normoxia were stained with Hoechst dye and assessed by flowcytometry. When EDCs and CDCs were incubated with 50 µM Verapamil, there were no side population cells in the designated area, indicating that EDCs and CDCs contain some side population cells. The proportions of side population cells in EDCs and CDCs were (0.8±0.2% and 0.45 ±0.1%, $p<0.05$), respectively. Higher fractions of these cells were observed in hypoxic EDCs and CDCs (1.8±0.2%vs 0.83±0.3%), respectively compared to their normoxia counterparts. The data clearly indicated that EDCs and CDCs contain side population cells and hypoxia increases their quantity (Figure 7).

In vivo angiogenic properties of EDCs and CDCs

Thus far, our data showed that the expression of stem cell (Sca-1,cKit and Abcg2), endothelial and angiogenic markers (Eng, Flk1, Vegf) increased significantly in EDCs and CDCs culture in hypoxia. We also observed that culturing EDCs and CDCs in hypoxia elevates the level of VEGF secreted into their media. Therefore, to assess all these *in vitro* benefits of hypoxic environment, we evaluated EDCs' and CDCs' pro-angiogenic effect (CD31 expression cells) *in vivo* with matrigel plug angiogenesis assay. EDCs and CDCs were derived from CAG-farnesyl-eGFP transgenic mice to enable the tracking of GFP-labelled cells in the matrigel plugs. This assay does not require surgical procedures and allows for the screening of the angiogenic properties of different cells. Matrigel is an extract of the Engelbreth-Holm-Swarm tumour and contains basement membrane proteins to support vessel formation. In this study (2×10^6) EDCs or CDCs cultured in normoxia or hypoxia were directly injected sub-cutaneously with growth factor reduced matrigel and after 10 days the effect of EDCs and CDCs on neovascularisation was assessed by detailed quantification of MVD (CD31 positive cells) in the matrigel using histochemistry staining. Comparison of MVD between two groups: normoxia CDCs (^{n}CDC) Figure 8 (A&E), versus hypoxia CDCs (^{h}CDC) Figure 8 (B&F), showed that matrigel plugs with ^{h}CDCs had significantly higher MVD levels compared to matrigel plugs with ^{n}CDC, (8± 2.4 ^{n}CDC vs 14.2± 4.4 ^{h}CDC, Figure 8I, P=0.001). Interestingly in EDCs, the hypoxia EDC (^{h}EDC) group, figure 8 (D&H) showed a higher MVD level in comparison with normoxia EDCs (^{n}EDC), Figure 8(C&D). (12.5± 1.8 ^{n}EDC/ vs 24.3±2.8 ^{h}EDC, Figure 8I, p=0.001). After comparing the angiogenic responses between CDCs and EDCs in both environments, EDCs showed better angiogenesis compared to CDCs. Our previous study at Newcastle University by tracking genetically labelled CDCs showed that very limited numbers of GFP labeled CDCs co-express CD31 (endothelial cell marker- Figure 8J) and α-SMA (smooth muscle marker- Figure 8K). In that we concluded that these cell contribute indirectly to neovessle formation mechanisms and mainly activate paracrine pathways (Figures 8J and 8K)[11].

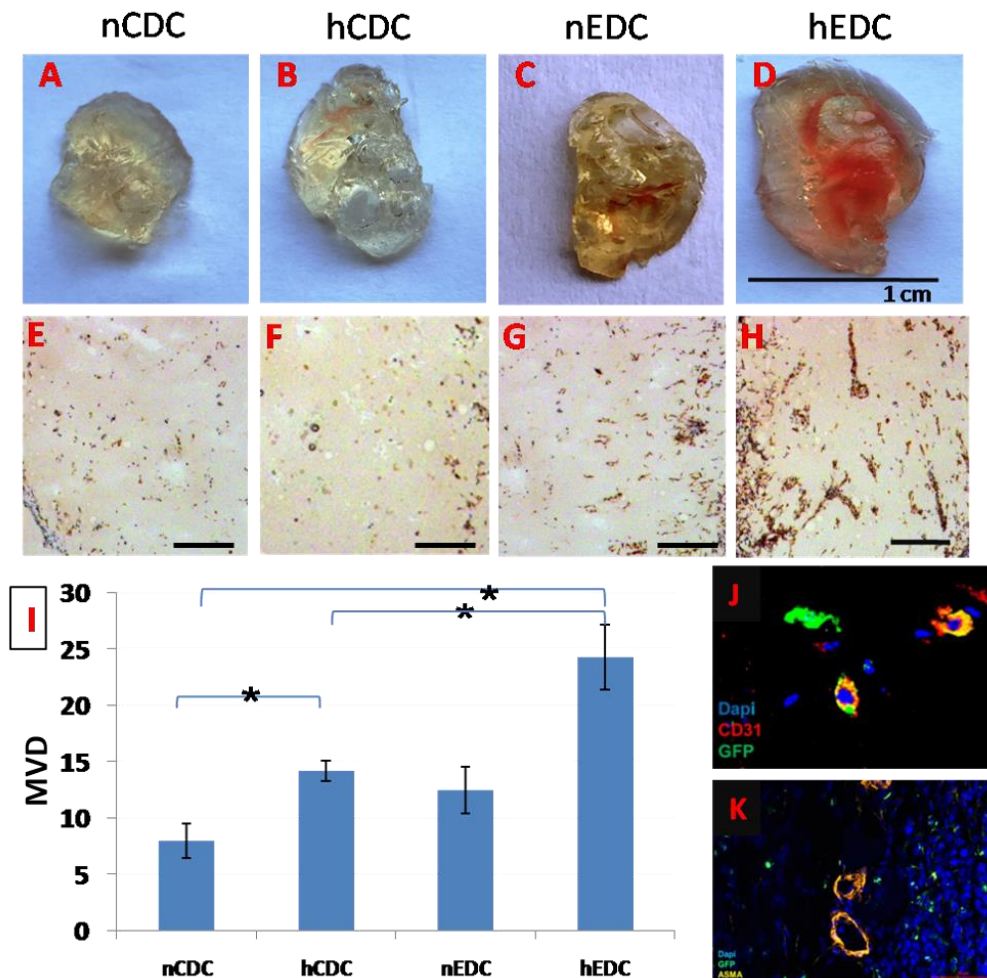

Figure 8: *In vivo* angiogenesis results from EDCs or CDCs cultured in normoxia and hypoxia, following sub-dermal matrigel plug injection. (A&E) matrigel plug from normoxia CDCs, (B&F) Hypoxia CDCs, (C&G) Normoxia EDCS, (D&H) Hypoxia EDCs, (I) The summary of microscopic MVD analysis from matrigel plugs, (J) co-localisation of GFP and CD31 and (K) ASMA in CDCs. J and K are modified from Amirrasouli, 2014[11]. *p=0.001, scale bar (E-H) = 500µm.

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Discussion

The aims of this study were to assess the stemness and angiogenic properties of EDCs and CDCs in normoxia and hypoxia. As CDCs and Csphs cultures are time consuming and expensive, here we assessed whether hypoxia preconditioning of EDCs could be used as the ultimate cell therapy method for cardiac repair studies. The novelty of our findings could be summarised as follows: first we showed the positive effect of hypoxia on EDCs outgrowth from cardiac explants, which is quite different from the previous studies[10, 14]. Previous studies used very low O_2 levels (0.1 %)[10], which could be very lethal for stem cells and might not be tolerable for long duration cell cultures such as EDCs. Li *et al*, (2011) used physiological O_2 (5%)[14], which might not be enough to activate hypoxia mediated pathways in order to enhance their pro-angiogenic and cardiogenic properties. As the final aim of cardiac progenitor cell studies is to provide an optimal cell type for cardiac repair, here we used 3% O_2,which according to Rumsey *et al*.(1994) recapitulates the O_2 level in the border zone region of the infracted heart[15].This region is the favoured target to deliver candidate cells for cardiac regeneration studies. Therefore if candidate cells are treated with 3% O_2, it is likely that they will be able to tolerate and accommodate themselves more properly than the cells that are treated with different O_2 concentrations.

In vitro studies

Our data showed that hypoxia increased EDC outgrowth significantly (1236±48.3 µm in normoxia vs. 2420.7±124µm in hypoxia, $p<0.05$) in comparison to normoxia. These data are in agreement with Li et al. (2011) who showed that EDCs grow faster in 5% O_2 than in normoxia. As hypoxia is shown to stimulate cell migration through the perk/atf4/lamp3 pathway[16] it is likely that hypoxia may increase EDC migration out of the explants through the same pathway. Detailed analysis of the cytokines involved in paracrine activities of EDCs could validate this finding. Here, we also showed that hypoxia increased the expression of the Stat3/Bclx pathway, and therefore, it is likely that these cells may be able to live longer than their normoxia control groups and could tolerate the hypoxic environment of the infarct region following cell transplantation. This is in agreement with Yokogami *et al*.(2013) and Selveniran *et al*.(2009), who showed the important effect of stat3 in hypoxic environments and the anti-apoptotic features of ovarian cancer cells[17, 18].Analysing the effect of hypoxia on CDC showed that culturing CDCs in hypoxia did not result in any significant difference in CDC proliferation, and this finding is consistent with and van Oorschot *et al*. (2011), who showed that hypoxic cell culture did not change cardiac progenitor proliferation at earlier stages, however, prolonged hypoxic culture (>6 days) increased cell proliferation and migration significantly[19]. Therefore, we can conclude that the effect of hypoxia on cell proliferation and apoptosis is cell- and context-dependent, and it is also possible to prolong CDC cultures and compare the proliferation rate at their higher passages.

It is already known that cardiac progenitor cells express cardiac stem cell markers such as cKit and Sca-1[20] and the benefit of hypoxic cell culture on human cardiac stem cells has been shown(21). Our data showed that EDCs cultured in hypoxia, express more stem cell markers than their normoxic counterparts and more than CDCs (Figure 5). Our findings showed that explants contain some progenitor cells that express stem cells and angiogenic factors, including Sca-1, cKit, Abcg2, Eng and Vegf, and if they are cultured in a hypoxic environment, these markers would be expressed more.

Our experiments on cardiac side populations by flowcytometry showed that both cell types (EDCs and CDCs) contain a fraction of side population cells and hypoxia increases these cells significantly ($^{nEDC}0.8 \pm 0.2$ vs $^{hEDC}1.8\pm0.2$/ $^{nCDCs}0.45\pm0.1$ vs ^{hCDC} 0.83 ± 0.3, $p<0.05$). Davis *et al.* (2010) showed that cardiac explant-derived cells could provide functional benefits equivalent to CDC[1]. In their work, EDCs were assessed by *in vitro* and *in vivo* experiments in order to compare their stemness, pro-angiogenic and cardiogenic properties with CDCs[1]. In general, they showed the feasibility of working with EDCs in comparison to CDCs.

In vivo studies

To validate our *in vitro* findings, we injected EDCs or CDCs derived from CAG-farnesylated-eGFP mice and cultured in normoxia or hypoxia. Then the cells were sub-dermally injected into mouse models and compared their angiogenesis through the quantification of CD31 expressing cells. We utilised 3% O_2 cell culture, a novel hypoxic preconditioning strategy in cardiac progenitor cell culture that is related to the hypoxic environment of the cardiac infarct border zone[15]. This is an important concept because the cardiac region of interest for cell delivery in clinical studies is the border zone. If the candidate cells are cultured with severe hypoxic conditions (0.0 - 0.2% O_2) and then delivered into the border zone, the new environment would be hyperoxic and conversely, if they are preconditioned with physiological O_2 (5-7% O_2), the infarct border zone may be hypoxic. Therefore, either hyperoxic or hypoxic environments could be harmful to the candidate cells and could alter their retention and cardiogenic properties. However, culturing the donor cells with 3% O_2 could mimic very similar conditions of the infarct border zone and could provide a better understanding of their performance.

Our *in vivo* model of angiogenesis showed that sub-dermal delivery of murine EDCs or CDCs combined with matrigel leads to a significantly enhanced CD31 expression within the matrigel and the EDCs' response was more evident in both normoxia and hypoxia. Previously our immunofluorescence staining with CAG-farnesyl-eGFP transgenic line showed that the majority of eGFP expressing CDCs do not co-localise with CD31-expressing cells, which suggests that the angiogenic response from the injected CDCs is likely to be paracrine (Figures 8J and 8K).

Our *in vivo* data is confirmed by the *in vitro* data as we showed that after culturing EDCs and CDCs in hypoxia, the level of cardiac stem cell marker Sca-1 was increased. Sca-1 is shown to be involved in neo-angiogenesis[22] and elevated VEGF expression under the control of HIF1-α from cells in a hypoxic environment is shown to be an early sign of angiogenesis[23]. In addition, the level of VEGF protein in the supernatant of EDCs and CDCs cultured in hypoxia was increased (2.4-fold in EDCs and 1.7-fold in CDCs).

In summary, we have shown that preconditioning of EDCs and CDCs with hypoxia, increases their cardiac stem cell, endothelial sub-populations and enhances their angiogenic capacity in sub-dermal matrigel assay. However, to elucidate the exact mechanisms, it will be important to investigate further the precise role of EDC- or CDC-mediated angiogenesis. More importantly, these findings suggest that sub-dermal injection of EDCs or CDCs act through paracrine mechanisms, and therefore, to understand the exact pathway, it would be advantageous to assess which proteins are involved by these cells.

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Abbreviations

α-SMA	α smooth muscle actin
CDC	Cardiosphere-derived cell
Csphs	Cardiospheres
CDCs	Cardiosphere derived cells
CHD	Coronary heart disease
DAB	3-3"-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified Eagle's medium
EDCs	Explant derived cells
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
Eng	Endoglin
EDTA	Ethylene-diamine-tetra-acetic acid
Flk-1	Foetal liver kinase 1
FACS	Fluorescent activated cell sorting
GFP	Green fluorescent protein
Hif-1α	Hypoxia inducible factor- 1-α
MI	Myocardial infarction
mRNA	Messenger RNA
PBS	Phosphate buffered saline
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Sca-1	Stem cell antigen
VEGF	Vascular endothelial growth factor

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Potential Conflicts of Interests

None

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