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Combination of Cardiac Progenitor Cells From the Right Atrium and Left Ventricle Exhibits Synergistic Paracrine Effects In Vitro

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Abstract

Cardiovascular diseases, such as ischemic heart disease, remain the most common cause of death worldwide. Regenerative medicine with stem cell therapy is a promising tool for cardiac repair. Combination of different cell types has been shown to improve the therapeutic potential, which is thought to be due to synergistic or complimentary reparative effects. We investigated if the combination of cardiac progenitor cells (CPCs) of right atrial appendage (RAA) and left ventricle (LV) that are isolated from the same patient exert synergistic or complimentary paracrine effects for apoptotic cell death and angiogenesis in an in vitro model. Flow cytometry analysis showed that both RAA and LV CPCs expressed the mesenchymal cell markers CD90 and CD105, and were predominantly negative for the hematopoietic cell marker, CD34. Analysis of conditioned media (CM) collected from the CPCs cultured either alone or in combination in serum-deprived hypoxic conditions to simulate ischemia showed marked increase in the level of pro-survival hepatocyte growth factor and pro-angiogenic vascular endothelial growth factor-A in the combined RAA and LV CPC group. Next, to determine the therapeutic potential of CM, AC16 human ventricular cardiomyocytes and human umbilical vein endothelial cells (HUVECs) were treated with CM. Results showed a significant reduction in hypoxia-induced apoptosis of human cardiomyocytes treated with CM collected from combined RAA and LV CPC group. Similarly, matrigel assay showed a significantly increased tube length formed by HUVECs when treated with CM from combined RAA and LV CPC group. Our study provided evidence that the combination of RAA CPCs and LV CPCs may have superior therapeutic effects due to synergistic paracrine effects for cardiac repair. Therefore, in vivo studies are warranted to determine if a combination of different stem cell types have greater therapeutic potential than single-cell therapies.

Keywords

cardiac progenitor cells, synergistic effect, paracrine secretion, hypoxia, apoptosis, angiogenesis

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality globally, with an estimated 17.9 million deaths annually. Ischemic heart disease (IHD), a condition characterized by the decreased blood supply to the heart, is the predominant cause of death in CVD accounting for 8.9 million deaths, approximately half (49.7%) of CVD deaths[1]. Current available treatment can delay progression toward heart failure but is unable to replace the lost cardiovascular cells, which is thought to be essential for long-term improvement of cardiac function in patients with IHD and heart failure (HF)[2].

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Stem cell therapy holds great promise for their potential to repair and regenerate cardiac tissue lost in IHD and HF. Regenerative therapy using stem cells provides a promising adjunct to current treatment with the potential to facilitate regeneration and repair of cardiac tissue by transplantation of stem cells. Current challenges in cell therapy include identifying the ideal stem cell source, optimal delivery method, and improving cell survival, retention, and engraftment of stem cells into cardiac tissue[3,4]. Among other cell types, cardiac progenitor cells (CPCs) were shown to have better effects on cardiac repair compared to other adult stem cell (ASC) populations and seem to be a promising candidate for cardiac repair[5]. Furthermore, a combination of different stem cell types that have synergistic or complementary effects may produce greater therapeutic effects than single-cell therapies[6]. Our recent study showed that different populations of stem cells in the heart have different functional qualities, with CPCs from the right atrial appendage (RAA CPCs) having better cell survival potential, and CPCs from the left ventricle (LV CPCs) having better angiogenic potential, which are major targets for cardiac repair[7]. Since studies have identified functional differences across different stem cell populations, a combination of cells that exert complementary or synergistic effects may have the potential for greater therapeutic effects on cardiac repair than single-cell populations.

Early evidence for the clinical relevance of combination cell therapy for cardiac repair was shown using combined transplantation of skeletal myoblasts with bone marrow mononuclear cells after myocardial infarction in rats[8]. Similarly, combined transplantation of skeletal myoblasts with $CD133^+$ cells in a model of chronic IHD improved cardiac function, reduced scar size and apoptosis, and increased angiogenesis in rats[9]. Skeletal myoblasts improve cardiac function by introducing contractile elements, while $CD133^+$ cells increase angiogenesis[9]. The functional benefits of the combined transplantation were mainly attributable to the increased neovascularization, which improved the blood supply for the engraftment of the skeletal myoblasts.

Initially, it was thought that the differentiation of stem cells was responsible for improvements seen in cardiac function. Pluripotent stem cells and CPCs can differentiate into cells of the cardiac lineage, although the potential of other ASCs to differentiate into cells of the cardiovascular lineage remains highly controversial[4,10]. Additionally, the functional improvements following stem cell transplantation are not sufficiently explained by differentiation due to the relatively small number of new cardiomyocytes formed[10,11]. This has led to a paradigm change, and it is now believed that the majority of the therapeutic effects of cell therapy is due to the paracrine-mediated impacts[12,13].

All these evidence suggest that despite the recent controversy, stem cell therapy is a promising therapy for regenerating the functional capacity of the diseased heart[14–16]. CPCs are involved in heart development, cell turnover, and endogenous repair following an injury to the heart, and hence are contenders as the ideal stem cell type[14,17]. Furthermore, a combination of different stem cells that have different reparative effects on the heart can be combined to further increase the efficacy of cell therapy[7,18]. To date, no studies are showing the combined effects of CPCs derived from different heart chambers of the same patient on the cardiac repair.

Therefore, this study aimed to evaluate the synergistic paracrine effects of a heterogeneous population of CPCs from the RAA and LV of the same patient on in vitro cardiac repair. RAA CPCs and LV CPCs were cultured alone or in combination, in serum deprivation in normoxic and hypoxic conditions. Conditioned media (CM) collected following serum deprivation were used for further in vitro experiments to determine the paracrine effects of the different CPCs. Results have provided evidence that the combination of RAA and LV CPCs may have greater therapeutic effects and have synergistic paracrine effects for cardiac repair.

Materials and Methods

Ethical Approval and Tissue Collection

The Health and Disability Ethics Committee in New Zealand approved the use of human left ventricular (LV) tissues (LRS/12/01/001/AM13). The RAA and LV tissues of patients ($n = 6$ each) with coronary artery disease who underwent coronary artery bypass grafting surgery in Dunedin hospital were collected following their informed consent. Cardiac tissue was collected in Krebs Ringer Henseleit solution, then transferred to a tube containing Hank's balanced salt solution (HBSS) and kept on ice until cell isolation.

Cell Culture

Cells used in this study were cultured according to the standard conditions as described previously[13,19]. CPCs isolation and culture conditions are detailed below. AC16 cardiomyocytes are a proliferating human cardiomyocyte cell line derived from adult human ventricular cardiomyocytes, which were fused with SV40-transformed, uridine auxotroph human fibroblasts devoid of mitochondrial DNA[20].

AC16 cardiomyocytes were cultured in Dulbecco's modified Eagle medium/F12 supplemented with 12.5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), and 1× anti-anti (Gibco, Waltham, MA, USA), according to the manufacturer's protocol.

Human umbilical vein endothelial cells (HUVECs, ATCC, Manassas, VA, USA) were cultured in endothelial growth medium 2 (EGM-2) (Lonza, New Zealand) containing endothelial basal medium 2 (EBM-2) supplemented with EGM-2 SingleQuots (Lonza, New Zealand). HUVECs were frozen using complete media supplemented with 10% FBS, and 10% dimethyl sulfoxide. HUVECs at passages 4 and 5 were used for the tube formation assay to determine angiogenic effects of the CM (detailed below).

Table 1. Clinical Characteristics of Study Participants.

			Diabetes										
Patient#	Age	Sex	Yes/ No	Duration (years)	HbA1c (mmol/mol)	BMI	Total cholesterol	Triglycerides	HDL	LDL	LVEF	E/A ratio	E/e′
237	61	M	No	–	33	30.9	2.3	1.2	1.40	0.4	44	1.15	9.71
421	65	M	No	–	36	28.9	5.3	2.3	1.18	3.1	58.5	0.66	7.09
436	62	M	No	–	39	27.1	3.9	1.0	1.23	2.2	53	0.83	10.57
493	64	F	No	–	40	26.4	4.5	1.4	1.66	2.2	60	0.77	11.67
550	64	M	No	–	35	23.7	4.7	0.6	1.75	2.7	50.5	2.59	6.54
552	57	M	No	–	33	22.4	2.8	0.8	0.99	1.4	66	0.98	5.94
Average	62.2	–	–	–	36	26.57	3.9	1.2	1.37	2.0	55.3	1.16	8.59
SD	2.67	–	–	–	2.71	2.89	1.06	0.55	0.27	0.89	7.10	0.66	2.17

BMI: body mass index; HbA1c: glycated hemoglobin; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LVEF: left ventricular ejection fraction.

Isolation and Culture of CPCs

CPCs were isolated from the RAA and LV tissue through cell dissociation using collagenase II and subsequent seeding under specific culture conditions, as previously described[13,19]. In brief, after removing the surrounding adipose tissue, RAA and LV tissue was washed twice with HBSS and minced into smaller pieces. The minced tissue was then placed into a 15-ml tube and allowed to settle on ice. The supernatant was aspirated, and 7 ml of fresh HBSS with 0.04% collagenase II solution was added and transferred to a T25 flask. The tissue was incubated in a shaker-incubator set at 160 rpm at 37°C for 60 min. Following incubation, the supernatant was collected in a fresh tube with 10% FBS and kept on ice. The remaining tissue was mechanically triturated by repetitive pipetting to separate the cells. The supernatant was then collected. Both the cell suspensions were mixed and centrifuged at 300 RCF for 5 min. The resulting pellet was resuspended in 1 ml Ham's F12 (Thermofisher, New Zealand) supplemented with 10 ng/ml human basic fibroblast growth factor (Sigma-Aldrich), 0.005 U/ml human erythropoietin (Sigma-Aldrich), 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), and 1× antibiotic-antimycotic (anti-anti) (Thermofisher, New Zealand).

Due to the variation in size between RAA and LV samples, cells were seeded onto an appropriately sized plate or flask based on the weight of the tissue to minimize differences in initial seeding density (Supplemental Table 1). The cells were then incubated in a 5% CO_2/95% air humidified incubator at 37°C for 24 h. Following 24 h, cells were supplemented with fresh culture media. Thereafter, culture media were changed every 2 to 3 days or subcultured when the cells become 90% confluent. The resultant CPCs were characterized by flow cytometry for the expression of mesenchymal stem cell (MSC) markers, CD90 (thymocyte differentiation antigen, Thy-1) and CD105 (endoglin), and the circulating hematopoietic progenitor cell marker CD34 as previously described[13,19]. To confirm the ability of CPCs to differentiate into cardiomyocytes in vitro, cells were grown with the commercially available cardiac differentiation medium (Millipore) according to the manufacturer's instructions. The expressions of cardiac troponin-T and Nkx2.5 were quantified using quantitative reverse transcriptase polymerase chain reaction (RT-PCR). For immunofluorescence, cells were fixed with freshly prepared 4% paraformaldehyde and stained with antibodies against cardiac myosin heavy chain kinase (MHC, Abcam, New Zealand) and connexin-43 (Abcam, New Zealand) as described earlier[7]. In addition, undifferentiated CPCs were also stained for CPC specific marker Islet-1 (Novus Biologicals). All the experiments were conducted with the cells between fourth and fifth passage.

Exposure of Serum-Deprived CPCs to Normoxic and Hypoxic Conditions

To mimic the ischemic environments as in IHD, cells were cultured in serum deprivation in hypoxic conditions (Supplemental Table 2). Normoxic conditions were used as a control. Cells were cultured either in single (RAA and LV) or in combination (RAA+LV) in a 5% CO_2/95% air humidified incubator at 37°C for 24 h to allow cells to adhere. Following 24 h, the culture media were changed to serum-free (SF) Ham's F12 media containing Ham's F12 nutrient mixture supplemented with 1× anti-anti. Cells in the normoxia group were cultured in 20% O_2/5% CO_2 humidified incubator for 72 h while cells in the hypoxia group were cultured in 1% O_2/5% CO_2 humidified incubator for 72 h.

Following the experimental period, culture media were collected in a 15-ml tube and centrifuged at 300 RCF for 5 min. The supernatant (conditioned media, CM) was collected, without disturbing the cell pellet, and aliquoted into sterile tubes. The CM was stored in a −80°C freezer for further experiments.

Following the collection of the CM, RNA was extracted from the cells as described earlier[21,22]. Extracted RNA was stored in a −80°C freezer until used for analysis.

Gene Expression Analysis in CPCs

Two hundred fifty nanograms of total RNA was reverse transcribed using Primescript RT reagent kit (Takara, Japan) according to the manufacturer's protocol. The resultant cDNA was amplified using polymerase chain reaction (PCR) master mix (Takara, Japan) with the specific primers for *HIF1A*, *AKT1*, *FGF2*, and *PDGFA* (Kicqstart Sybergreen Primers, Sigma-Aldrich, New Zealand), which encode for the proteins HIF-1α, AKT1, bFGF, and PDGF-A, respectively. HIF-1α and AKT1 are involved in cell signaling pathways in cardioprotection, angiogenesis, and differentiation. bFGF and PDGF-A are secreted by CPCs to mediate their therapeutic effects for cardiac repair[11]. 18 S was used as a reference gene and was added for every plate used. The data are represented as relative DCT expression.

Enzyme-Linked Immunosorbent Assay for Quantification of Growth Factors in CM and Cell Lysate

Our previous study showed abundant release of pro-survival insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) and pro-angiogenic vascular endothelial growth factor (VEGF) by both RAA and LV CPCs[7]. All these 3 paracrine factors secreted by stem cells have been shown to exert cardioprotective and angiogenic effects respectively[7,11]. Therefore, for the current study, these three factors were chosen to be quantified in the CM of all the study groups. IGF-1 and VEGF-A were purchased from Elisakit.com, Australia and HGF was purchased from eBioscience (Thermofisher, New Zealand). Assay was conducted according to the manufacturer's protocol. Results were calculated using GraphPad Prism 8 according to the manufacturer's instructions.

Effect of CM on Cardiomyocytes Cultured Under Hypoxic Conditions

The effects of the CM on apoptosis and activation of transcription factor HIF-1α in AC16 cardiomyocytes treated under hypoxic condition was measured using a Caspase-Glo 3/7 assay and enzyme-linked immunosorbent assay, respectively, as previously described[7]. For this, AC16 cardiomyocytes were seeded onto a 96-well plate at 3,000 cells/well in triplicates. After 24 h, the culture media were replaced with new media consisting of 50 µl of CM collected from RAA, LV, and RAA and LV combined CPCs grown in normoxia or hypoxia and 50 µl of SF Ham's F12. Cells were then incubated in hypoxia (1% O_2) for 72 h.

At the end of the experimental period, culture media were replaced by 25 µl of PBS and 25 µl of caspase reagent. Contents of the well were gently mixed on a plate shaker set at 250 rpm for 1 min, and the plate was incubated at room temperature for 30 to 60 min, and luminescence was measured using a plate reader. Following the caspase assay, CyQUANT reagent (Thermofisher, New Zealand) was prepared at a concentration of 2× and 50 µl was added to each well for a final concentration of 1×. The plate was then incubated at room temperature in the dark for 10 min, and the fluorescence was measured using a plate reader set at excitation at 480 nm and emission at 520 nm. Caspase activity (relative luminescent units) was then normalized to cell number (relative fluorescence units) (caspase activity/cell number) before analysis and represented as fold changes to the cells grown in complete growth media in normoxia.

Angiogenic Effects of CM on HUVECs Using Tube Formation Assay

The angiogenic potential of the CM was measured in HUVECs using a tube formation assay. For this, HUVECs were seeded onto the Matrigel-coated 96-well plate in triplicates (18,000 cells per well in 50 µl of SF EBM-2). Thereafter, 50 µl of CM was added to the treatment wells. For the negative control, an additional 50 µl of SF EBM-2 was added. For the positive control, HUVECs in 100 µl EGM-2 complete media were seeded in triplicates (18,000 cells per well). The plate was then incubated in a 5% CO_2/95% air humidified incubator at 37°C for 12 h.

At the end of the experimental period, phase-contrast images at 4× magnification were taken using a microscope (Olympus, Japan) fitted with a microscope camera (Lumenera, USA). Images were then analyzed using the software Fiji using the angiogenesis analyzer plugin to quantify total tube length[23].

Statistical Analysis

Differences in cell marker expression between RAA CPCs and LV CPCs, gene expression, and IGF-1, VEGF-A, and HGF concentrations were analyzed using two-way analysis of variance (ANOVA), followed by a Tukey's post hoc test. Caspase/CyQUANT data were normalized to the Ham's F12 complete media group. Two-way ANOVA was performed to compare the SF and CM-treated groups against the control F12 complete media, followed by Dunnett's post hoc test. Differences in the total tube length from the tube formation assay of the CM-treated groups were compared against the positive control EGM-2 complete media using one-way ANOVA followed by a Dunnett's post hoc test. Data represented as mean ± SEM. Differences with a P-value ≤ 0.05 were considered statistically significant.

Results

Patient Characteristics

The medical history and echocardiographic reports were collected from all patients before surgery (Table 1). Information such as age, sex, medical history, and echocardiogram report were available for each patient. All parameters were comparable across all the patients, except one

patient having a reduced ejection fraction (<50%) (patient number 237).

Comparable expression of cell surface markers between RAA CPCs and LV CPCs

CPCs were characterized for the expression of CD34, CD90, and CD105. The expression of cell markers was comparable between RAA CPCs and LV CPCs, and there were no statistical differences.

Majority of the cells were negative for CD34 population, although due to the ischemic nature of the tissue collected they still expressed CD34, which was especially more in LV CPCs (18.3% ± 3.3% in RAA CPCs vs. 24.7% ± 6.0% in LV CPCs, $P = 0.23$, $n = 6$ patients, Fig. 1A, C).

CD34 negative population were then used to measure the expression of MSC markers, CD90 and CD105. CD90 expression was significantly higher in RAA CPCs compared to LV CPCs (78.7% ± 5.9% in RAA CPCs vs. 54.4% ± 11.0% in LV CPCs, $P = 0.03$, $n = 6$ patients, Fig. 1B, D). CD105 expression was high in both RAA CPCs and LV CPCs, with nearly all cells expressing the cell marker (99.%0 ± 0.6% in RAA CPCs vs. 99.3% ± 0.1% in LV CPCs, $P = 0.2505$, $n = 6$ patients, Fig. 1B, D). Due to the high expression of CD105, the $CD90^{+}CD105^{+}$ cells were almost entirely dependent on CD90 expression (76.8% ± 5.0% in RAA CPCs vs. 54.6% ± 11.1% in LV CPCs, $P = 0.06$, $n = 6$ patients, Fig. 1B, D). Further, immunocytochemical analysis confirmed the expression of cardiac transcription factor Islet-1 in the undifferentiated CPCs (Fig. 1E).

Differentiated CPCs Expressed Adult Cardiomyocytes Markers

CPCs grown in differentiation medium showed positive staining for cardiomyocyte-specific markers MHC (Fig. 1F) and connexin-43 (Fig. 1G). RT-PCR analysis confirmed the expression of cardiac troponin and α-sarcomeric actin in differentiated CPCs (Fig. 1H). As expected, there was no difference in the differentiation potential between RAA and LV CPCs.

Decreased Hypoxia Inducible Factor-1A mRNA Expression in Hypoxia

CPCs were exposed to hypoxia to mimic in vitro ischemic conditions. In contrary to the expectation, hypoxia significantly reduced the expression of hypoxia inducible factor-1A (*HIF1A*) gene in CPCs from all the groups ($P < 0.05$ hypoxia vs. normoxia for all the groups, Fig. 2A). There was no statistically significant difference in mRNA expression of *AKT1*, *FGF2,* and *PDGFA* between any of the CPC groups (Fig. 2B-D). Importantly, we did not find any synergistic effects following co-culture with RAA and LV CPCs.

Hypoxia Increased Secretion of Pro-Survival HGF and VEGF-A Into the CM

Next, the CM collected at the end of the hypoxic period was used to measure the secretion of pro-survival HGF and pro-angiogenic VEGF-A concentration. Hypoxia significantly increased the secretion of VEGF-A in both RAA and RAA + LV CPC group (Fig. 3A). Importantly, RAA + LV CPC group showed a further increase in VEGF-A concentration following hypoxia, suggesting a synergistic pro-angiogenic effect (Fig. 3A). Similarly, hypoxia also increased the concentration of HGF in the CM collected from CPCs exposed to hypoxia. However, the level was comparable between all the study groups with no significant difference observed with RAA and LV combined CPCs (Fig. 3B). Interestingly, another pro-survival growth factor IGF-1 showed a decreased trend in all hypoxic groups. However, this was significant only in the LV CPCs exposed to hypoxia compared to the corresponding normoxia group (Fig. 3C).

CM From CPCs Reduce Hypoxia-Induced Apoptosis in Human Cardiomyocytes

Next, we determined whether increased HGF has any effect on hypoxia-induced apoptotic cell death in AC16 human ventricular cardiomyocytes. As expected, AC16 cardiomyocytes cultured under the hypoxic condition without CM showed a significant increase in caspase-3/7 activity, suggesting increased apoptotic cell death (Fig. 4A). Interestingly, all the CM-treated cells showed a decrease in caspase-3/7 activity, although this effect was significant only in the cells treated with CM collected from both normoxic and hypoxic combined CPCs (Fig. 4A). Further, there was a significant reduction in caspase-3/7 activity in cells treated with CM from combined RAA and LV CPCs cultured in hypoxia over LV or RAA CPC CM alone (Fig. 4A). These results together demonstrate the synergistic effects of combined cell therapy. Comparison of apoptotic effects of normoxic and hypoxic CM irrespective of the groups did not show any significant difference (Fig. 4B).

Improved Angiogenesis of HUVECs Treated With CM From RAA and LV Combined CPCs

Finally, the effect of CM to improve the angiogenic potential of endothelial cells was estimated by measuring the tube-forming ability of HUVECs (Fig. 5A). All the CM-treated groups showed a significant improvement in angiogenesis compared to the negative control SF EBM-2 group (Fig. 5A, B).

Cells treated with CM collected from the RAA + LV CPCs exposed to hypoxia showed higher tube length compared to all the other groups, and this was significant against CM collected from RAA CPCs and combined RAA and LV CPCs cultured in normoxia (Fig. 5A, B). Importantly, the tube length in combined RAA and LV CPCs combined

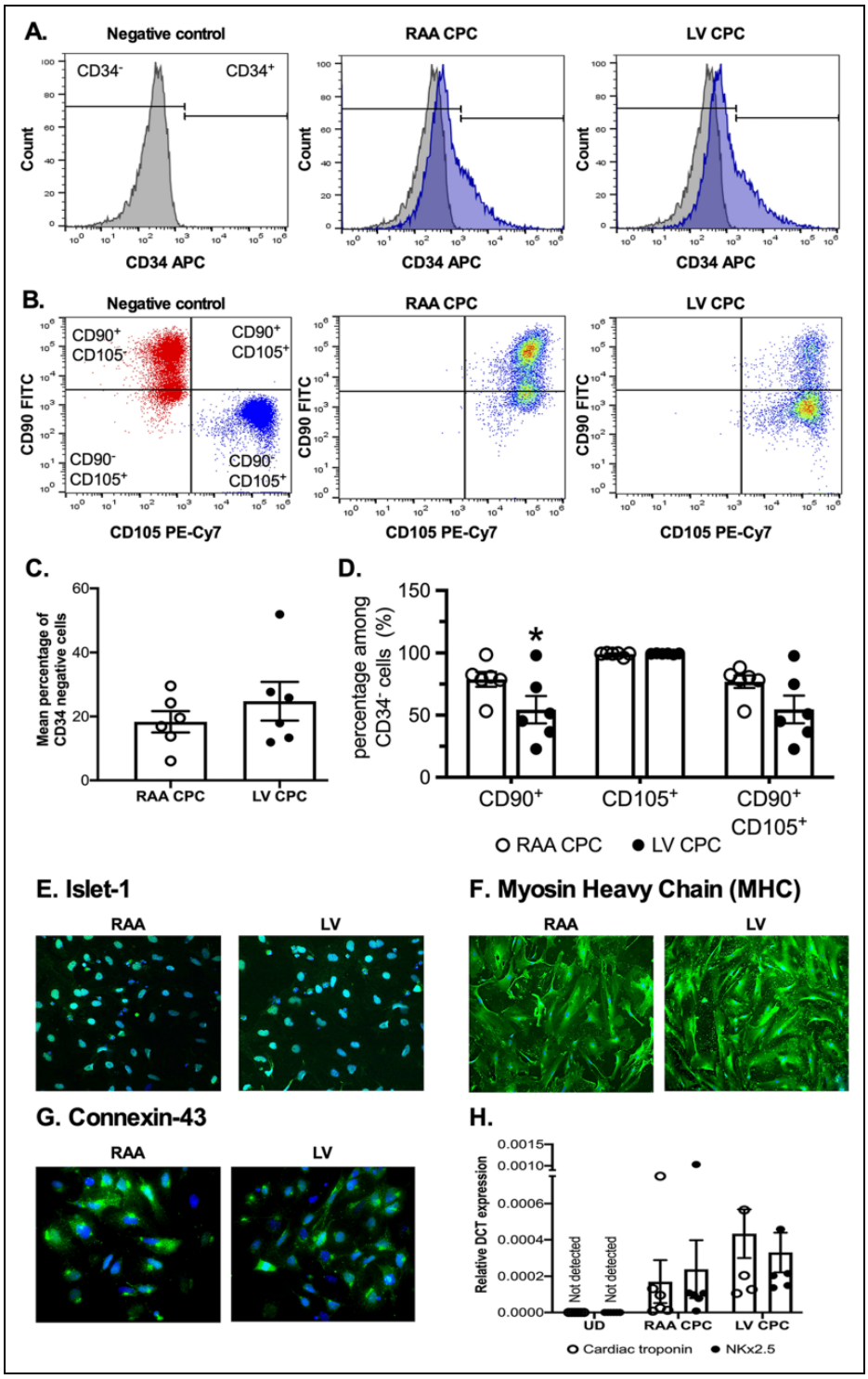

Fig. 1. A. Representative histograms showing the expression of CD34 in RAA and LV CPCs. B. Representative bivariate dot plots showing the expression of CD90 (*y* axis) and CD105 (*x* axis) within the $CD34^{-}$ population in RAA and LV CPCs. C. Quantitative scatter plot bar graph showing the expression of $CD34^{+}$ population in RAA and LV CPCs. D. Quantitative scatter plot bar graphs showing the expression CD90, CD105, and CD90 CD105 double positive populations in $CD34^{-}$ population in RAA and LV CPCs ($n = 6$ each). Data represented as mean $\pm$ SEM %. Differences between RAA CPCs and LV CPCs in CD34 population were analyzed by paired *t*-test and difference in the population of CD90 and CD105 was analyzed by two-way analysis of variance followed by Tukey's post hoc test. E-G. Representative fluorescent microscopy images showing the expression of cardiac transcription factor Islet-1 in undifferentiated CPCs (E) and adult cardiomyocytes marker MHC (F) and connexin-43 (G). H. Quantitative scatter plot bar graphs showing the expression of adult cardiomyocytes marker cardiac troponin and Nkx2.5. $n = 6$ in each group. CPC: cardiac progenitor cell; FITC: fluorescein isothiocyanate; LV: left ventricle; MHC: myosin heavy chain; PE: phycoerythrin; RAA: right atrial appendage .

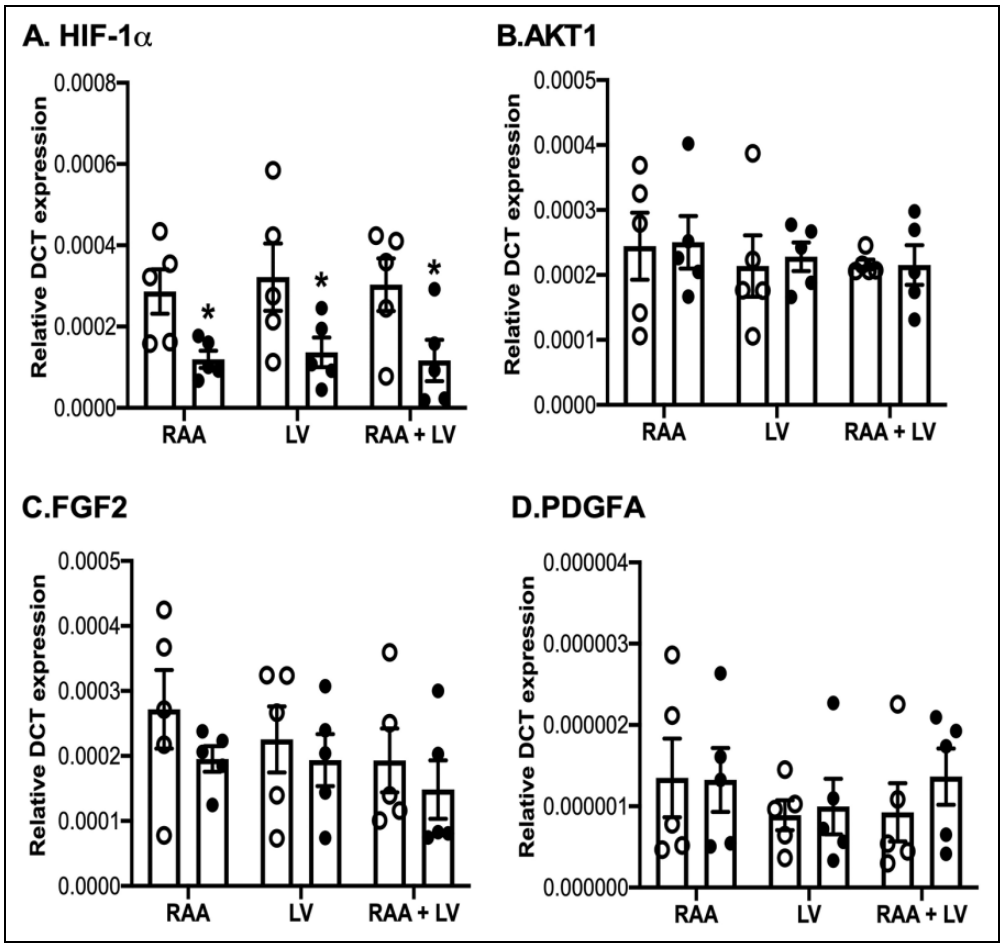

Fig. 2. Gene expression of CPCs following serum deprivation in normoxic and hypoxic conditions. Quantitative scatter plot bar graphs showing mRNA expression *HIF1A* (A), *AKT1* (B), *FGF2* (C), and *PDGFA* (D), respectively ($n = 5$ patients, triplicates) in RAA and LV CPCs cultured alone or in combination under normoxic and hypoxic conditions. Data are mean $\pm$ SEM and represented as relative delta cycle threshold (DCT) expression. Differences were compared using two-way analysis of variance followed by a Tukey's post hoc test; $^*P < 0.05$ vs. corresponding normoxia group. CPC: cardiac progenitor cell; HIF1A: hypoxia inducible factor 1A; LV: left ventricle; RAA: right atrial appendage.

group was comparable to the positive control EGM-2 complete media-treated group (Fig. 5A, B).

Interestingly, comparison of angiogenic effects of normoxic and hypoxic CM irrespective of the groups showed a significant increase in tube formation by hypoxic CM (Fig. 5C).

Discussion

Our study provides evidence that the combination of RAA CPCs and LV CPCs may have greater therapeutic effects in cardiac repair due to synergistic paracrine effects. This was demonstrated by significantly reduced apoptotic cell death in cardiomyocytes, and improved angiogenic potential of endothelial cells that were treated with CM collected from RAA and LV combined CPCs that are exposed to hypoxia. The major strength of this study is the comparison of CPCs isolated from the same patient; hence, all the experiments were performed using patient-matched samples and at matched passages, avoiding interpatient variations.

Flow cytometry analysis showing the presence of CD34 in our cell population was not surprising, provided that all the samples were collected from patients with myocardial ischemia, which could have mobilized the $CD34^+$ cells from the bone marrow into the heart[24,25]. While CD34 was primarily thought to be a specific marker for hematopoietic cells, studies have also demonstrated the existence of $CD34^+$ cells in the heart as cardiac endothelial precursors, which, in addition to CD34, also express the cardiac cell marker, homeobox gene *NKX2.5*[26,27]. Therefore, $CD34^+$ cells in our study could be of cardiac origin. Further, Messina et al. showed the presence of $CD34^+$ cells in cardiospheres, which are derived from a heterogeneous population of CPCs[28]. Similarly, CPCs isolated from the RAA and LV tissue in our

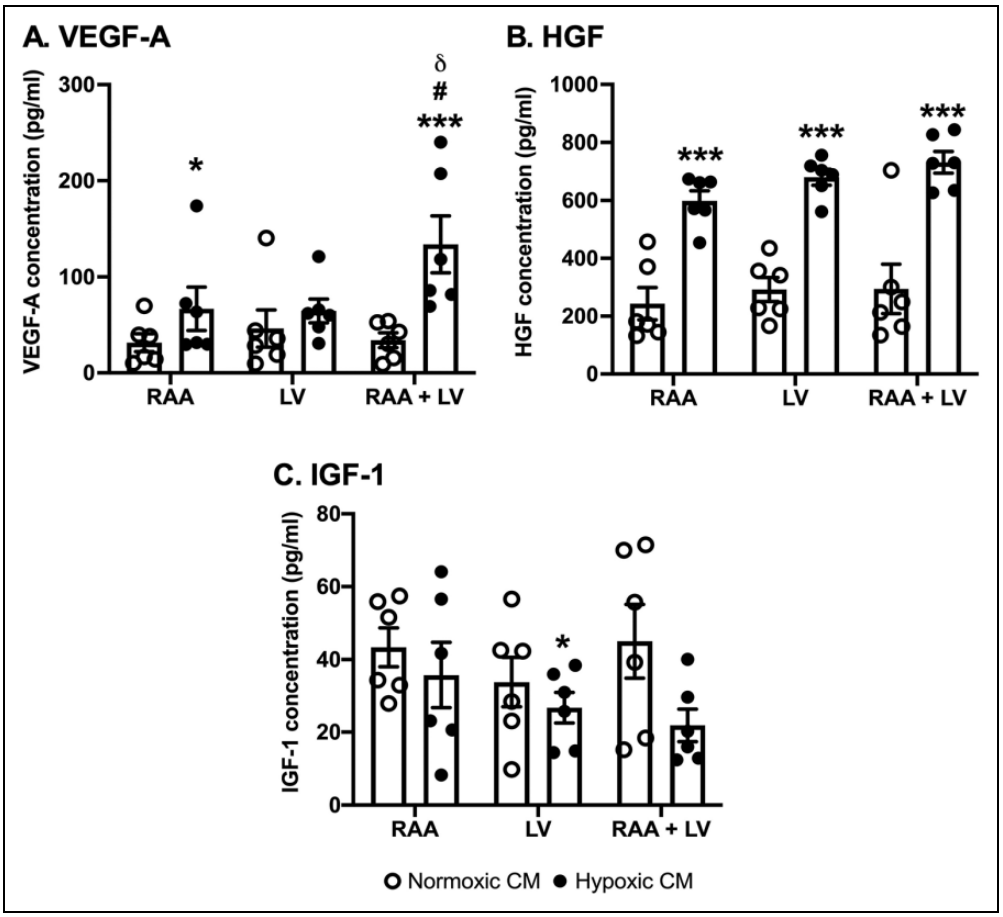

Fig. 3. Concentration of pro-angiogenic and pro-survival growth factors secreted by normoxic and hypoxic CPCs. Quantitative scatter plot bar graphs showing the concentration of pro-angiogenic VEGF-A (A) and pro-survival hepatocyte growth factor (HGF, B) and IGF-1 (C) secreted in the CM of RAA and LV CPCs cultured alone or in combination in normoxic and hypoxic conditions ($n = 6$ patients, duplicates). Data are mean $\pm$ SEM. Differences were compared using two-way analysis of variance followed by a Turkey's post hoc test. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. corresponding normoxic CM group; $^{\#}P < 0.05$ vs. hypoxic RAA CM; $^{\delta}P < 0.05$ vs. hypoxic LV CM. CM: conditioned media; CPC: cardiac progenitor cell; IGF-1: insulin-like growth factor-1; LV: left ventricle; RAA: right atrial appendage; VEGF-A: vascular endothelial growth factor A.

study contain a heterogeneous population of cells including cells from the epicardium and endocardium. Therefore, the presence of $CD34^{+}$ cells in our population is inevitable.

An unexpected finding in this study was the decreased *HIF1A* gene expression after exposing the CPCs to hypoxia. Studies have shown a significant increase in *HIF1A* gene expression following acute hypoxia (at 4 h); however, prolonged hypoxia ($\geq$12 h) showed a decrease in *HIF1A* expression[29]. Several mechanisms have been proposed for this decrease in *HIF1A* gene following prolonged hypoxia through negative-feedback mechanisms to prevent excessive accumulation of HIF-1α. One such mechanism is the upregulation of antisense HIF-1α and miR-429, both of which are increased in hypoxia[29,30]. Additionally, repressor element-1 silencing transcription factor represses *HIF1A* gene expression in prolonged hypoxia[31]. Further, tristetraprolin, an mRNA destabilizing protein, is also implicated in *HIF1A* mRNA destabilization following hypoxia[32]. In our study, CPCs exposed to prolonged hypoxia could have activated these factors to reduce *HIF1A* gene expression.

Interestingly, the decrease in *HIF1A* gene expression in hypoxia contradicted the level of VEGF-A concentration in the CM, which was highest in the CM collected from RAA and LV combined CPC group. VEGF-A is upregulated in hypoxia through the transcriptional action of HIF-1α. However, studies have shown HIF-1α-independent mechanism for activation of VEGF-A. Rasmussen et al. demonstrated protease-activated receptor-mediated activation of VEGF-A in human adipose stem cells[33]. Similarly, Pore et al. showed Akt-mediated induction of VEGF-A in astrocytes through a HIF-1α-independent mechanism[34]. In support of this, CPCs exposed to hypoxia-induced activation of Akt[35,36]. Therefore, in spite of reduced *HIFA* mRNA expression, CPCs in our study possibly activated VEGF-A via Akt-mediated pathway.

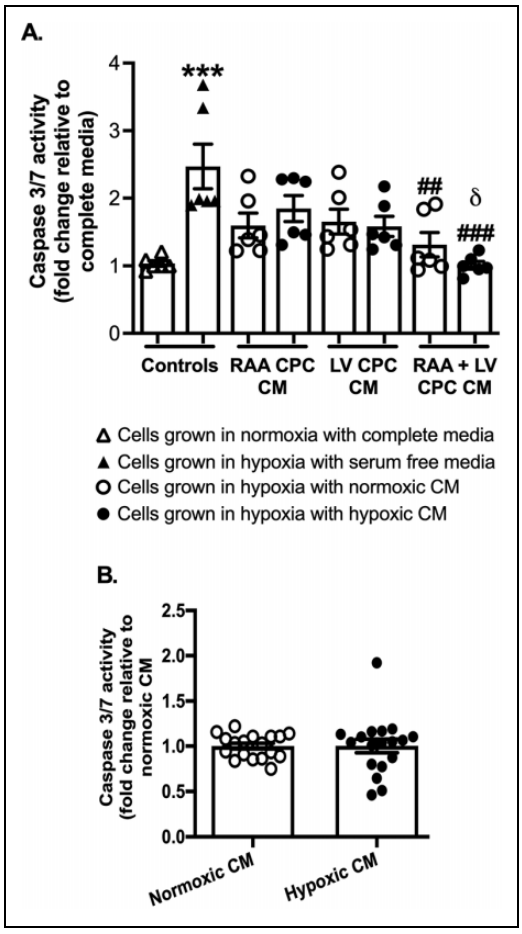

Fig. 4. Effect of CPC CM on hypoxia-induced human cardiomyocytes apoptosis. A. Quantitative scatter plot bar graphs showing caspase-3/7 activity in AC16 human ventricular cardiomyocytes cultured in hypoxic conditions and treated with CM from RAA and LV CPCs and combined RAA and LV CPCs cultured in normoxic and hypoxic conditions ($n = 6$ patients, triplicates). Data are mean $\pm$ SEM and represented as fold change relative to cells grown in complete media in normoxia. $^{***}P < 0.001$ vs. cardiomyocytes grown in normoxia with complete media; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. cardiomyocytes grown in hypoxia with serum free media; $^{\delta}P < 0.05$ vs. cardiomyocytes grown in hypoxia with CM from LV CPCs exposed to hypoxia. B. Quantitative scatter plot bar graphs showing the difference in caspase-3/7 activity of cardiomyocytes cultured in normoxic and hypoxic CM irrespective of cell types.CM: conditioned media; CPC: cardiac progenitor cell; LV: left ventricle; RAA: right atrial appendage.

HGF has been demonstrated to have a major role in cardiomyogenesis, and its expression is detectable as early as embryonic day 7.5 in the heart[37]. HGF is essential for self-repair of the heart after myocardial infarction MI[38], and the level of circulating HGF is significantly increased in patients following acute MI[39]. Similarly, our study showed a marked increase in HGF level in the CM collected from CPCs exposed to hypoxia. There was no further additive increase in the RAA and LV combined CPC group. On the contrary, the concentration of the other pro-survival growth factor IGF-1 was decreased in all the CM collected from CPCs exposed to hypoxia. While there was no previous evidence for the hypoxia-induced reduction in IGF-1 concentration in CPCs, CM collected from osteoblasts had reduced IGF-1 concentration, due to the decrease in Runt-related transcription factor 2 in hypoxia, an osteoblast transcription factor that can activate an upstream response element in the IGF-I gene promoter[40]. Similarly, in vascular endothelial cells, IGF-1 expression was decreased in hypoxia through the actions of insulin-like growth factor binding protein, a group of carrier proteins for IGF-1 that modulate IGF-1 activity, which can be both stimulatory or inhibitory[41]. Although these mechanisms are likely to be cell-specific, c-Kit^{+} CSC/CPCs have been shown to differentiate into osteogenic and vascular lineages[42]. Therefore, it is possible that similar mechanisms could play a role in reducing IGF-1 concentration in CPCs exposed to hypoxia.

The most striking finding of this study was a marked improvement in cardiomyocytes survival and endothelial cells' angiogenic potential under hypoxic conditions after treatment with the CM. Importantly, these effects were markedly increased with the CM collected from the RAA and LV combined CPC group. The previous study has shown that the antiapoptotic effects of RAA hypoxic CM were mediated through the PI3K/AKT and PKCε pathways, while LV hypoxic CM mediated these effects through the PKCε pathway[7]. In our study, the antiapoptotic effects of CM from RAA and LV combined CPCs were likely mediated through both pathways in addition to increased HGF, thus producing synergistic effects on cell survival. The angiogenic effects observed in our study correspond to increased VEGF-A secretion into the CM. However, it was interesting to observe improved angiogenesis in the CM collected from LV CPCs exposed to hypoxia, which had the lowest VEGF-A concentration in the CM. This is likely through the secretion of other angiogenic factors by LV CPCs. Previous studies have demonstrated activation of pro-angiogenic platelet endothelial cell adhesion molecule-1[43] and angiopoietin-like 4[44] in CPCs exposed to hypoxia. Although we have not measured these factors in the current study, it is logical to postulate that some of these factors could have been responsible for VEGF-independent angiogenesis in LV CPCs.

In conclusion, results from this study have provided evidence that combination therapy may provide synergistic effects; hence, identification of the effective combination of stem cells that exert synergistic and complementary effects on cardiac repair could be more beneficial. Combination of RAA and LV CPCs synergistically improved HGF and VEGF-A secretion, and improved survival of cardiomyocytes and angiogenic potential of endothelial cells by paracrine mechanisms. Stated as part of the "next-generation" of cell therapy, combination of stem cells is likely to play an important role in the future of stem cell therapy. While the results of the study are appealing, the technical difficulties and invasiveness of procuring different stem cell population also need

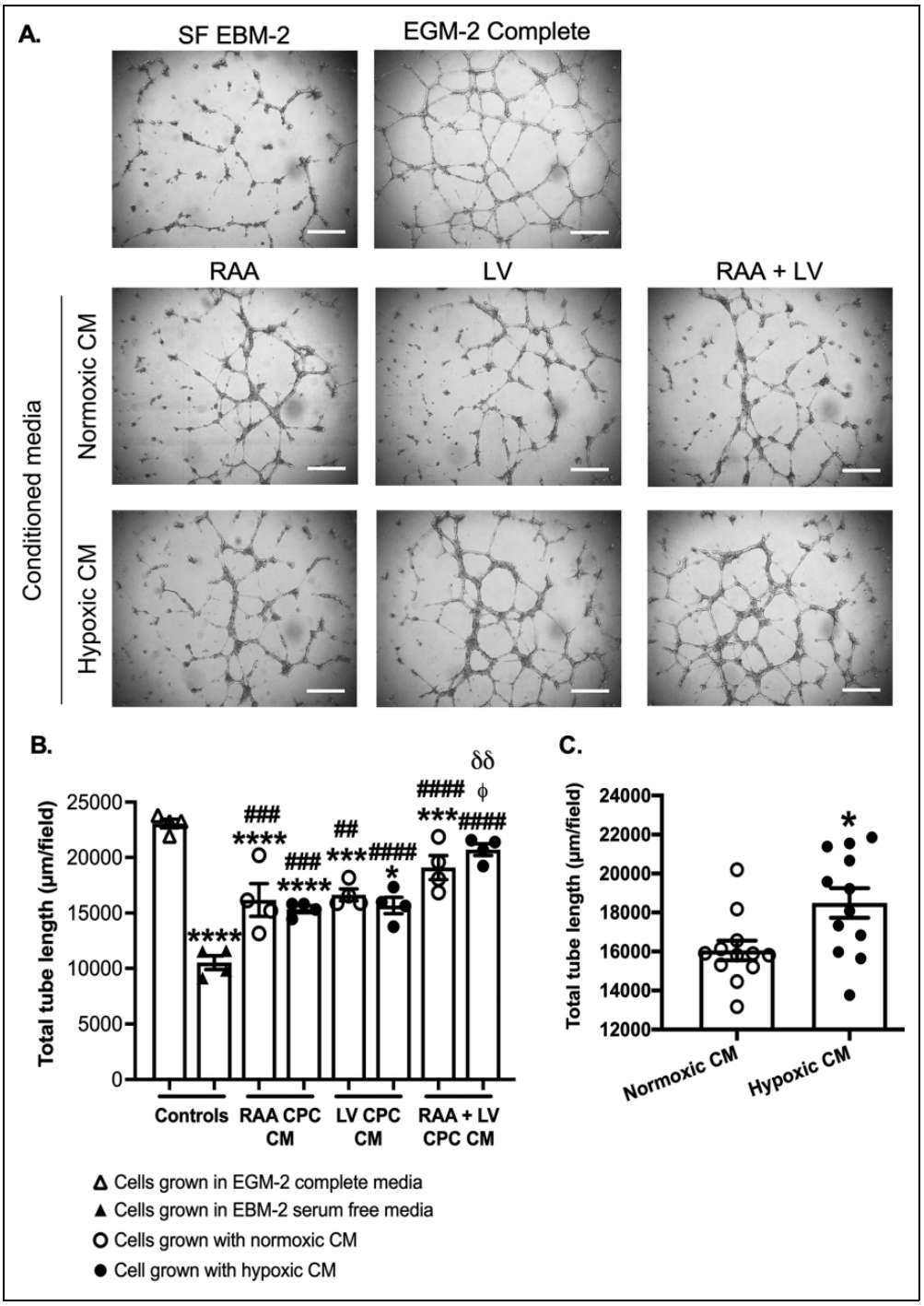

Fig. 5. Angiogenic effects of CM on tube formation of HUVECs. A. Phase-contrast images showing tube formation in HUVECs taken at 4× magnification 12 h after initial seeding. Scale bars, 400 µm. B. Quantitative scatter plot bar graphs showing the tube length in HUVECs cultured with CM from RAA and LV CPCs and combined RAA and LV CPCs grown in normoxic and hypoxic conditions ($n = 6$ patients, triplicates). *$P < 0.05$, ***$P < 0.001$ and ****$P < 0.0001$ vs. cells grown in EGM-2 complete media; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ vs. cells grown in EBM-2 serum free media; $^{\delta\delta}P < 0.01$ vs. cells grown in CM from hypoxic RAA CPCs; $^{\phi}P < 0.05$ vs. corresponding normoxic CM-treated cells. C. Quantitative scatter plot bar graphs showing the difference in tube length formed by HUVECs cultured in normoxic and hypoxic CM irrespective of cell types. CM: conditioned media; CPC: cardiac progenitor cell; EBM-2: endothelial basal medium 2; HUVEC: human umbilical vein endothelial cell; LV: left ventricle; RAA: right atrial appendage; SF: serum-free.

to be assessed and compared against the therapeutic benefits of combination therapy.

Authors Contribution

All authors contributed to the interpretation of data and reviewing of the manuscript. RM performed all the experimental work and data analysis; PD established the CPC isolation and culture; AY contributed to CPC isolation and flow cytometry analysis; IVH maintained the patient database; JBP performed PCR analysis of differentiated CPCs. RD, DP, and PD collected the human tissue samples; RK conceived, designed, and supervised the study, and wrote the final version of the manuscript.

Authors' Note

Dr Dixit is now with the Drug Discovery Unit, School of Life Sciences, University of Dundee, Dundee, UK

Data Availability

All the data from this study are included in the manuscript.

Ethical approval

The Health and Disability Ethics Committee in New Zealand approved the use of human left ventricular tissues (LRS/12/01/001/AM13).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with The Health and Disability Ethics Committee in New Zealand (LRS/12/01/001/AM13)-approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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