Cardiac atrial appendage stem cells promote angiogenesis in vitro and in vivo

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1 **Cardiac Atrial Appendage Stem Cells Promote Angiogenesis *in vitro* and *in vivo***

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20 **CASCs Promote Angiogenesis**

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ABSTRACT

Cardiac atrial appendage stem cells (CASCs) show extraordinary myocardial differentiation properties, making them ideal candidates for myocardial regeneration. However, since the myocardium is a highly vascularized tissue, revascularization of the ischemic infarct area is essential for functional repair. Therefore, this study assessed if CASCs contribute to cardiac angiogenesis via paracrine mechanisms.

First, it was demonstrated that CASCs produce and secrete high levels of numerous angiogenic growth factors, including vascular endothelial growth factor (VEGF), endothelin-1 (ET-1) and insulin-like growth factor binding protein 3 (IGFBP-3). Functional *in vitro* assays with a human microvascular endothelial cell line (HMEC-1) and CASC CM showed that CASCs promote endothelial cell proliferation, migration and tube formation, the most important steps of the angiogenesis process. Addition of inhibitory antibodies against identified growth factors could significantly reduce these effects, indicating their importance in CASC-induced neovascularization. The angiogenic potential of CASCs and CASC CM was also confirmed in a chorioallantoic membrane assay, demonstrating that CASCs promote blood vessel formation *in vivo*.

In conclusion, this study shows that CASCs not only induce myocardial repair by cardiomyogenic differentiation, but also stimulate blood vessel formation by paracrine mechanisms. The angiogenic properties of CASCs further strengthen their therapeutic potential and make them an optimal stem cell source for the treatment of ischemic heart disease.

Keywords: angiogenesis; growth factors and cytokines; stem cell; cardiac progenitor cells; endothelial cell

1 ABBREVIATIONS AND ACRONYMS

ALDH	aldehyde dehydrogenase
ANG	angiogenin
Ang-1	angiopoietin-1
CAM	chorioallantoic membrane
CASC	cardiac atrial appendage stem cell
CM	conditioned medium
CSC	cardiac stem cell
DAPI	4′,6-diamidino-2-phenylindole
DPPIV	dipeptidyl peptidase 4
DSC	dental stem cell
EC	endothelial cell
GM-CSF	granulocyte-monocyte colony stimulating factor
HMEC-1	human microvascular endothelial cell line 1
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cell
ET-1	endothelin 1
FBS	fetal bovine serum
IGFBP-1/2/3	insulin-like growth factor binding protein 1/2/3
IL-8	interleukin 8
MCP-1	monocyte chemotactic protein 1
MI	myocardial infarction
MSC	mesenchymal stem cell
MTT	3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Neg Contr	negative control
Pos Contr	positive control
P/S	penicillin/streptomycin

PTX-3	pentraxin 3
TIMP-1	tissue inhibitor of metalloproteinase 1
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor

1. INTRODUCTION

Ischemic heart disease is still one of the major causes of global morbidity and mortality since current therapies are not able to repair the damaged heart muscle [4]. Recent developments in stem cell biology and regenerative medicine show promise to replace the lost myocardium with functional healthy tissue [27]. Various stem cell types such as induced pluripotent stem cells [38], mesenchymal stem cells (MSCs) [18] and cardiac stem cells (CSCs) [2, 40] have shown potential to improve heart function after myocardial infarction (MI). Most beneficial effects observed so far were mediated by paracrine actions, as stem cells secrete cytokines, growth factors and miRNAs that promote cardioprotection, angiogenesis and activate resident CSCs. However, only moderate therapeutic effects were observed in clinical trials. This can be explained by limited differentiation of these stem cells towards cardiomyocytes [15].

Recently, our research group identified a new CSC type in the adult human heart based on high aldehyde dehydrogenase (ALDH) enzyme activity, known as the cardiac atrial appendage stem cell (CASC) [22]. These CASCs are able to preserve left ventricular function in a Göttingen minipig infarction model based on extensive cardiomyogenic differentiation and functional integration [12, 22]. Moreover, CASCs can be expanded to clinically relevant cell numbers [42], making them a perfect candidate for myocardial regeneration. However, to fully restore cardiac function, revascularization of the infarcted tissue is essential. Current catheter-based interventions and surgical bypass procedures are often not successful in reestablishing myocardial blood flow in MI patients, leading to an increased mortality and a poor clinical outcome [3]. In addition, cells transplanted in the infarct area end up in an ischemic environment, implicating that new blood vessel formation is necessary for their successful survival, engraftment and differentiation [36]. Ideally, cell-based therapies should therefore focus on both cardiomyogenesis and angiogenesis to induce optimal repair of the heart muscle. This study explores a possible contribution of CASCs in myocardial angiogenesis by investigating expression of pro- and anti-angiogenic factors and their role in three consecutive steps of the angiogenesis process (endothelial proliferation, migration, tube formation). Stimulation of

1 angiogenesis would further strengthen the therapeutic potential of the CASCs in ischemic heart
2 conditions.

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2. MATERIALS AND METHODS

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the institutional review board and informed consent from each patient were obtained.

2.1 Cell culture

Atrial appendages were obtained from ischemic heart disease patients undergoing routine cardiac surgery and CASCs were isolated from atrial appendages based on a high ALDH enzyme activity as previously described [22]. Heart tissue was dissociated by collagenase type II treatment (600U/mL; Invitrogen) and the obtained single cell suspension was stained with Aldefluor® (STEMCELL Technologies Inc.) according to the manufacturers' instructions. ALDH+ cells were directly flow-sorted in X-vivo 15 supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The isolated CASCs were seeded in fibronectin-coated culture plates and expanded in X-vivo 15 medium supplemented with 20% FBS and 2% P/S. The medium was changed twice weekly and cells were re-plated at a density of $5x10^3$cells/cm² when reaching 80-85% confluence. After the first passage, serum levels were reduced to 10% and the cells were expanded.
A human microvascular endothelial cell line (HMEC-1) was used to study angiogenesis *in vitro* [19] and was obtained from the Centre for Disease Control and Prevention (Atlanta, GA). HMEC-1 were cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA) supplemented with 2% penicillin/streptomycin (P/S), 10 mM L-glutamine (L-glut), 10% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor (hEGF, Gibco, Paisley, UK) and 1µg/ml hydrocortisone (HC, Sigma-Aldrich, Diegem, Belgium).

2.2 Preparation of conditioned medium and cell lysates

CASC conditioned medium (CM) and lysates were prepared from CASC cultures of passage 3 to 7, when reaching 85-90% confluence. After washing with phosphate buffered saline (PBS), the cells

were cultured for 48h in serum-free low glucose Dulbecco's modified eagle medium (LG-DMEM) 2% P/S 0% FBS. CM was harvested and passed through a 0.22µm filter to remove cell debris. Afterwards the medium was concentrated in 3kDa Amicon Ultra-15 centriprep tubes YM-10 (Amicon, Millipore Corp.) at 3600g and sterile filtered to obtain 10X concentrated CM. Batches of 20X concentrated CM were also prepared for the *in vivo* chorioallantoic membrane (CAM) assay. In this way, a final concentration of 10X CM after 1:1 dilution in Matrigel was obtained. CASC viability was assessed after preparation of CM with an annexin V Kit to guarantee the quality of the CM (Number of viable CASCs >80%; Supplemental material). After CM collection, cell lysates were prepared by homogenization of CASCs in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail (1:100; Thermo Fischer Scientific, Erembodegem, Belgium) and stored at -80°C until further analysis.

10X or 20X concentrated LG-DMEM 2% P/S 0% FBS served as negative control medium (Neg Contr) and LG-DMEM 2% P/S 10% FBS as positive control medium (Pos Contr), unless stated otherwise. For the functional HMEC-1 assays, CASC CM was pre-incubated with 500ng/ml VEGF (R&D systems), 1µg/ml IGFBP3 (R&D systems), 100ng/ml ET-1 (Abcam) neutralizing antibody or isotype controls (ISO, R&D systems).

2.3 Human Angiogenesis Array

A Proteome Profiler™ Human Angiogenesis Antibody Array (R&D Systems, Minneapolis, MN) was performed on CASC CM of 3 different patients and on negative and positive control media to identify angiogenic factors according to the manufacturer's instructions. Signal detection was performed using the LI-COR Odyssey Infrared Imaging System in combination with IRDye® 800CW streptavidin (Li-Cor, Westburg, The Netherlands). Quantification was performed with LI-COR Odyssey Image Studio analyzer software. Data were normalized against the positive control spots (Positive control was set to 100%, Blue demarcations in Fig 1A).

2.4 Immunofluorescence for angiogenic growth factors

CASCs were cultured on glass coverslips and fixed in 4% paraformaldehyde (PFA) at 80% confluence. The cells were incubated overnight with a rabbit polyclonal anti-VEGF (1:100; R&D systems), a rabbit anti-IGFBP-3 (1:100; R&D systems) or a mouse anti-ET1 (1:100; Abcam) antibody at 4°C in PBS 0.3% Triton. An Alexa Fluor 594 goat anti-rabbit antibody or an Alexa Fluor 555 goat anti-mouse antibody (1:500; life technologies) was used as secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Pictures were taken with a Nikon Eclipse 80i fluorescent microscope and a Nikon DS-2MBWc digital camera.

2.5 Western blot

After heat denaturation, protein lysates were separated on 4-15% Protean® TGX™ Gels and transferred to 0.2µm Transblot® Turbo™ nitrocellulose membranes with the Transblot® Turbo™ Transfer System (all from Bio-rad, Temse, Belgium). Blots were blocked in Odyssey blocking buffer (Li-Cor) and primary antibodies used for western blot analysis included a rabbit anti-ET1 antibody (1:500; Abcam), a rabbit anti-IGFBP-3 antibody (1:300; Santa Cruz Biotechnology, Heidelberg, Germany) and a rabbit anti-VEGF antibody (1:300; Santa Cruz Biotechnology). A mouse anti-alpha-tubulin antibody (1:5000, Abcam) was used for the loading control. Secondary antibodies were a goat polyclonal anti-rabbit IR-Dye 800CW and a goat polyclonal anti-mouse IRDye 680CW antibody (1:15000; Li-Cor). Detection was performed using an Odyssey Infrared Imaging System (Licor).

2.6 Enzyme-linked immunosorbent assay

The concentration of identified angiogenic factors was determined by ELISA for the negative and positive control, CASC CM and CASC lysates. Quantikine ELISA kits against ET-1, IGFBP-3 and VEGF (R&D Systems) were used according to the manufacturer's instructions.

2.7 Proliferation assay

The effect of CASC CM on HMEC-1 proliferation was explored in a 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and by Ki-67 immunofluorescence analysis.

HMEC-1 were seeded at a density of $5x10^3$ cells/cm^2 in standard HMEC-1 culture medium. The following day, cells were washed with PBS and incubated with control media or CASC CM. After 72 hours, HMEC-1 proliferation was assessed. For the MTT assay, HMEC-1 cells were incubated with 5 mg/ml MTT (Sigma-Aldrich, Diegem, Belgium) in negative control medium. After an incubation period of 4 hours at 37°C, the MTT solution was removed and a mixture of 0.01 M glycine and DMSO was added to each well. Absorbance was measured at a wavelength of 540 nm with the FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). For the Ki-67 analysis, immunofluorescence was performed as described above with a Rabbit Ki-67 Ready-To-Use primary antibody solution (Thermo Fischer Scientific), which was diluted 1:1 in PBS with a final concentration of 0.3% Triton .

2.8 Migration assay

HMEC-1 migration was examined in a Transwell migration assay. The lower compartment consisted of 24-well plates containing the control media or CASC CM. Subsequently, HMEC-1 cells were seeded on 8-µm pore Thincert™ tissue culture inserts (Greiner Bio-One, Wemmel, Belgium) in the negative control at a density of $5x10^4$ cells/cm^2 (upper compartment). After 24h, the inserts were washed with PBS, fixed with 4% PFA and stained with 0.1% crystal violet. Representative pictures were taken with an Axiovert 200 M microscope (Carl Zeiss NV-SA, Zaventem, Belgium). Quantification of the area occupied by migrating HMEC-1 was performed by Axiovision 4.8 software (Carl Zeiss). Values were expressed as mean area percentage.

2.9 Tube formation assay

HMEC-1 tube formation was investigated by culturing HMEC-1 on phenol red-free Matrigel™ (Beckton & Dickinson) in 15µ angiogenesis slides (Ibidi, München, Germany). HMEC-1 were seeded on Matrigel at a density of $4x10^5$ cells/cm^2 in CASC CM or the negative and positive control. Standard HMEC-1 culture medium served as a positive control instead of LG-DMEM 2% P/S 10% FBS as the latter showed limited tube formation. Overview pictures were taken after 24 hours with an Axiovert 200 M microscope. Total tube length was quantified using the automated Angiogenesis Analyzer Tool for Image J [8].

2.10 Chorioallantoic membrane assay

The angiogenic properties of the CASCs were examined *in ovo* in the CAM assay [5, 19]. Fertilized white leghorn chicken eggs (Gallus gallus) were incubated at 37 °C in a humidified atmosphere. After 3 days (E3), 3–4 ml albumen was removed and a small opening was made in the shell. This opening was covered with cellophane tape and the eggs were returned to the incubator. After 6 days (E9), droplets of 30µl phenol red-free growth factor-reduced Matrigel™ (Amsbio, Abingdon, U.K.) were placed on the CAM. Droplets containing 50 000 CASCs were compared with pure Matrigel droplets as a negative control (Neg Contr 1). On the other hand, Matrigel mixed 1:1 with 20X CASC CM or 20X LG-DMEM 2% P/S 0% FBS (Neg Contr 2) was analyzed. On E12, the CAM was carefully removed from the eggs and pictures were taken. To quantify angiogenesis, two concentric circles (radii 3 and 4 mm) were drawn and intersecting blood vessels were counted by 2 independent researchers in a double-blind fashion. The assay was performed 3 times on CASCs and with CM from 6 different donors.

2.11 Statistical analysis

Data are reported as mean±SEM. Statistical analyses were performed with SAS version 9·3 software (SAS Institute Inc.). To take into account the different levels of dependency in the dataset, linear mixed models (proc MIXED in SAS) were fitted for all datasets with the different test conditions as fixed effects. For the functional *in vitro* assays, experiment and CM sample nested within experiment were fitted as random effects. Experiment was included as random effect to take variability between experiments into account. CM sample was included as random effect parameter since a paired relation exists between CM and CM with antibodies of the same patient sample. For the CAM assay only experiment was modeled as random effect. The different test conditions are compared using CONTRAST statements. For the functional HMEC-1 assays, a Bonferroni correction for multiple comparisons was implemented based on relevant comparisons between the test conditions of the functional assays. A global significance level of 5% is considered. Bonferroni correction for the multiple comparisons of the functional HMEC-1 assays resulted in a p-value of 0.004.

3. RESULTS

3.1 CASCs secrete numerous angiogenic growth factors

To investigate a possible paracrine effect of CASCs on angiogenesis, we first determined their expression and secretion of important angiogenic growth factors. Numerous anti- (red) and pro-angiogenic (green) factors were identified in CASC CM by a human angiogenesis array, whereas no growth factors were detected in the negative control that was not conditioned by CASCs (Fig 1a). Based on consistent high relative expression levels, the pro-angiogenic growth factors ET-1, IGFBP-3 and VEGF were further investigated. ELISA revealed high concentrations of all three factors in CASC CM, while the levels for the negative and positive control were below detection limit or neglectible (Fig 1b). CASC lysates contained substantially lower growth factor concentrations (Fig 1b) compared to CM. Expression of ET-1, IGFBP-3 and VEGF in CASCs was further confirmed by immunofluorescence in the cells (Fig 1c) and Western blot in lysates (Fig 1d). CASCS thus express and secrete factors that can positively influence the angiogenesis process.

3.2 CASCs promote HMEC-1 angiogenesis *in vitro* by the secretion of ET-1, IGFBP-3 and VEGF

Next we explored the effects of CASC CM on main steps of the angiogenesis process. It was demonstrated that the different test conditions did not affect HMEC-1 viability (Fig S2), confirming that effects on HMEC-1 survival did not negatively influence the results. Subsequently, the effects of CASC CM on HMEC-1 proliferation, migration and tube formation were studied. Involvement of the individual factors ET-1, IGFBP-3 and VEGF was assessed by pre-incubating CASC CM with inhibitory antibodies against these factors. CM incubated with isotype control antibodies (ISO) was included to rule out any non-specific influences. In all assays, serum-free medium that was not conditioned by CASCs served as negative control medium (Neg Contr). Medium containing 10% serum as positive control medium (Pos Contr), except for the tube formation assay where HMEC-1 culture medium served as positive control.

EC proliferation was investigated in an MTT assay and by Ki-67 immunofluorescence. HMEC-1

proliferation was increased after incubation with the positive control and CASC CM compared to the negative control in both the Ki-67 (Fig 2a,b; p-value 0.0004 and 0.0006 respectively) and the MTT assay (Fig 2c; p-value <0.0001 and 0.0038 respectively). Inhibition of VEGF reduced the effect of CASC CM in the Ki67 assay (Fig 2b; p-value <0.0001), while for the MTT assay only combined inhibition of ET-1, IGFBP-3 and VEGF led to a significant decrease (Fig 2c; p-value 0.0032). However, a clear trend was observed for all inhibitory antibodies in both assays and significance was reached while omitting the bonferoni correction.

To study the role of CASC CM in EC migration, a transwell migration assay was performed (Fig 3). Our results showed that HMEC-1 migration was 4.3 times higher for CASC CM compared to the negative control (p-value <0.0001), although the response was not that strong as the positive control. Incubation of the CM with antibodies against ET-1, IGFBP-3 or VEGF alone significantly diminished these effects (p-value <0.0001 for all antibodies), with an even stronger response when all three inhibitory antibodies were combined, reducing the migration effect of CASC CM by 2.7 fold (p-value <0.0001).

EC assembly into tube-like structures was explored by plating HMEC-1 on Matrigel in control media or CASC CM (Fig 4). HMEC-1 network formation, expressed as the total tube length, was significantly higher after culturing the cells in the positive control and CASC CM compared to the negative control (p-value 0.0001 and 0.0018 respectively). Pre-incubation of CASC CM with a combination of inhibitory antibodies against ET-1, IGFBP-3 and VEGF significantly reduced HMEC-1 network formation (p-value <0.0001), which could not be accomplished by blocking these growth factors individually.

These *in vitro* experiments show that CASCs promote all important steps of the angiogenesis process. The effect seems to be mainly mediated by ET-1, IGFBP-3 and VEGF secretion, although other factors are likely also involved.

3.4 CASCs promote angiogenesis *in vivo* in the chorioallantoic membrane assay

To confirm the angiogenic effect of both CASCs and CASC CM, an *in vivo* CAM assay was performed by incubating the CAM with CASCs, CASC CM or negative control samples (Fig 5a Neg Contr 1 = Matrigel; Neg Contr 2 = Matrigel mixed 1:1 with 20X LG-DMEM 2% P/S 0% FBS). After 72h, radial growth of blood vessels in a spoke wheel pattern towards the droplets was visible for all conditions (Fig 5b,c). Quantification of the number of blood vessels, based on intersection with two concentric circles (Fig 5b), revealed a significant increase in blood vessels for both CASCs and CASC CM compared to their negative control (Fig 5d). The number of blood vessels increased from 22.4 to 26.3 (inner circle; p-value 0.0364) and from 29.2 to 37.0 (outer circle; p-value 0.0011) for the negative control compared to CASCs respectively. For CASC CM an augmentation was observed from 18.9 to 23.3 (inner circle; p-value 0.0102) and from 29.1 to 32.5 (outer circle; p-value 0.0202) respectively. These results indicate that both CASCs and CASC CM promote blood vessel formation *in vivo*.

4. DISCUSSION

We have previously shown that CASCs, as one of the few CSC types, preserve cardiac function in a minipig MI model based on extensive engraftment and cardiomyogenic differentiation [12]. However, not only replacement of the lost heart muscle, but also new blood vessel formation is essential to restore cardiac function. This ensures the supply of critical oxygen and nutrients to the ischemic heart tissue, and guarantees the survival and engraftment of the transplanted progenitor cells [3]. Vasculogenesis and angiogenesis are the main mechanisms for new blood vessel formation, while arteriogenesis enhances blood flow by increasing the luminal diameter of existing arteries [7]. Vasculogenesis by EC differentiation and vascular integration has been described for e.g. endothelial progenitor cells (EPCs) [25], MSCs [20] and CSCs [2]. In contrast, EC differentiation was only rarely observed (<1%) after CASC transplantation in a minipig MI model, which is in agreement with the strong cardiomyogenic potential of CASCs. Regardless of this observation, an increase in blood vessel density in ischemic areas with transplanted CASC was observed, suggesting a role for CASCs in myocardial angiogenesis [12]. Hence, in line with the emerging paracrine hypothesis for stem cell-induced myocardial repair, possible paracrine angiogenic effects of CASCs were explored in this study.

Angiogenesis, characterized by the formation of new blood vessels via the branching or elongation of preexisting vessels, is tightly regulated by a balance of both pro- and anti-angiogenic mediators [7]. Numerous angiogenic inducers could be identified in CASC CM after an initial screening. ET-1 [37], IGFBP-3 [16] and VEGF [6, 28] were selected for further analysis, because of high expression levels in CASC CM. Their concentration in CASC lysates was consistently lower compared to the CM, as previously reported for MSCs [31]. This might already suggest a strong paracrine angiogenic potential for CASCs by the secretion of high levels of angiogenic proteins. The concentration of VEGF, as most widely studied angiogenic growth factor, is markedly higher in CASC CM compared to other stem cells types [19, 24]. However, direct comparison of growth factor concentrations in stem cell CM is not feasible, because of differences in the preparation of CM.

The importance of VEGF, IGFBP-3 and ET-1 in neovascularization further confirms a likely

involvement of these mediators in CASC-induced myocardial angiogenesis. VEGF is one of the most studied angiogenic factors and is involved in almost all steps of the angiogenesis process [6, 28]. IGFBP-3 has been reported to have inhibitory effects in tumor angiogenesis [26], while pro-angiogenic effects have been shown in human umbilical vein ECs [16]. ET-1 is a mitogen for vascular cells and promotes EC migration and tube formation [37]. Despite high relative expression levels of ANG and urokinase plasminogen activator (uPA), these factors were not investigated here as no suitable blocking antibodies were available. The role of all identified growth factors in angiogenesis can be found in Table S2. A wide variety of angiogenic growth factors are known to be secreted by various stem cell types, such as fibroblast growth factor, hepatocyte growth factor, interleukins and VEGF [6, 10, 29, 30]. Other reparative processes have also been shown to be influenced by these secreted growth factors, which also seem likely for the identified CASC secreted mediators. Indeed, VEGF reduces inflammation and promotes cardioprotection [6], while MCP-1 attracts monocytes and macrophages, which clear the infarct area from dead cells and debris leading to activation of reparative pathways [13].

Undoubtedly, the identified factors are involved in important steps of angiogenesis and therefore *in vitro* assays with HMEC-1 were performed to establish the functional pro-angiogenic effects of CASCs. EC proliferation is one of the first important steps in neovascularization and can best be investigated by a combination of cell number quantification and cell cycle analysis [39]. Both our experiments indicated that CASC CM stimulates an increase in HMEC-1 proliferation. Neutralization of VEGF reduced the effect of CASC CM in the Ki67 assay (Fig 2B; p-value <0.0001), while for the MTT assay only combined inhibition of ET-1, IGFBP-3 and VEGF led to a significant decrease (Fig 2C; p-value 0.0032). Discrepancy between the observed results can be explained by the different approach of the assays to assess proliferation. The Ki-67 assay quantifies the number of actively proliferating cells, while the MTT is a measure of the total number of cells and their metabolic activity. Still, a clear trend towards a reduction in HMEC-1 proliferation was observed for all inhibitory antibodies in both assays and significance could be reached for all antibody conditions without Bonferroni correction. Bonferroni correction is known to reduce the statistical power of the

test significantly [14]. Our data hence suggest ET-1, IGFBP-3 and VEGF to be important mediators of CASC-induced HMEC-1 proliferation. The proliferative effect of CASC CM was reduced to baseline levels in most antibody conditions. This clearly demonstrates the importance of these specific mediators in the delicate balance of pro- and anti-angiogenic proteins. Similar effects on EC proliferation have also been reported for CM obtained from various MSC types *in vitro* [11, 21, 35] and fetal aorta–derived CD133+ progenitor cells *in vivo* [1], while no proliferative effect was observed for dental stem cells (DSCs) [5, 19].

After the proliferation phase, ECs migrate towards chemotactic stimuli. In a transwell system, we were able to show that CASC CM induced similar HMEC-1 migration as the positive control. Individual inhibition of ET-1, IGFBP-3 and VEGF reduced this process, which was enhanced when combining the three inhibitory antibodies. This demonstrates that ET-1, IGFBP-3 and VEGF are all important mediators of CASC-induced EC chemotaxis. Despite this promising result, the effect of CASC CM was not completely reduced. This suggests that other factors are also involved in EC chemotaxis as shown in migration assays for EPCs [41], MSCs [11, 35], DSCs [5, 19] and other CSC types [43]. As a last step in the angiogenesis process, migrating ECs assemble into tube-like structures that form the base of the newly formed blood vessels, which was assessed in a Matrigel tube formation assay. Our results indicated that CASC CM improved HMEC-1 network formation as previously reported for MSCs [17], DSCs [5, 19] and other CSCs [9]. Only combined inhibition of ET-1, IGFBP-3 and VEGF reduced HMEC-1 tube formation to baseline levels, pointing towards a synergistic effect. Moreover, a dose-dependent effect on HMEC-1 tube formation was demonstrated as shown in supplementary figure S3.

Although CASCs stimulate the main phases of neovascularization, the formation of functional blood vessels requires successful occurrence of all subsequent steps of the angiogenesis process with complex interactions between ECs, pericytes or smooth muscle cells and stromal cells. Therefore, a CAM assay was performed to confirm the angiogenic effects of the CASCs *in vivo*. The CAM is a highly vascularized extraembryonic membrane which serves as a gas exchange surface of the chicken embryo. As the chick immune system is not fully established until late stages of development, the

embryo serves as a naturally immunodeficient host capable of sustaining grafted mediators and cells without species-specific restrictions [33]. Moreover, the CAM can be easily manipulated and observed, making it an ideal model to study angiogenesis *in vivo* [34]. Both CASCs and CASC CM promoted angiogenesis as observed by extensive radial ingrowth of blood vessels. Although CASC CM was concentrated 10X, similar effects were observed for CASCs and CASC CM. This can be explained by the continuous production of angiogenic growth factors by CASCs, while CM or single protein administrations require higher doses because of a short protein half-life [32]. Similar results were observed in CAM assays with DSCs [5, 19] bone marrow stromal cells [17] and placental MSCs [23].

In conclusion, CASC, isolated from atrial appendages of MI patients, stimulate angiogenesis *in vitro* and *in vivo*. This effect was mediated by the secretion of numerous growth factors that promote important steps of new blood vessel formation. Together with their strong cardiomyogenic differentiation potential, these newly identified angiogenic properties distinguish CASCs from other stem cells types. This unique therapeutic combination makes CASCs an interesting candidate for the treatment of ischemic heart disease. Translation from bench to bedside still requires more research to confirm the therapeutic effects of CASCs in a clinically relevant animal model with an isolation and expansion protocol suited for human application.

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7. DISCLOSURES

The authors report no relationships that could be construed as a conflict of interest.

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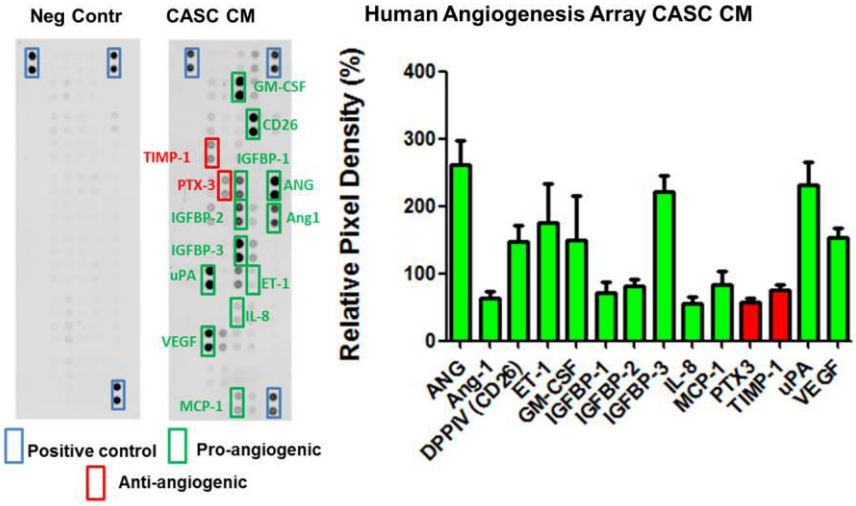
1 9. FIGURES

a.



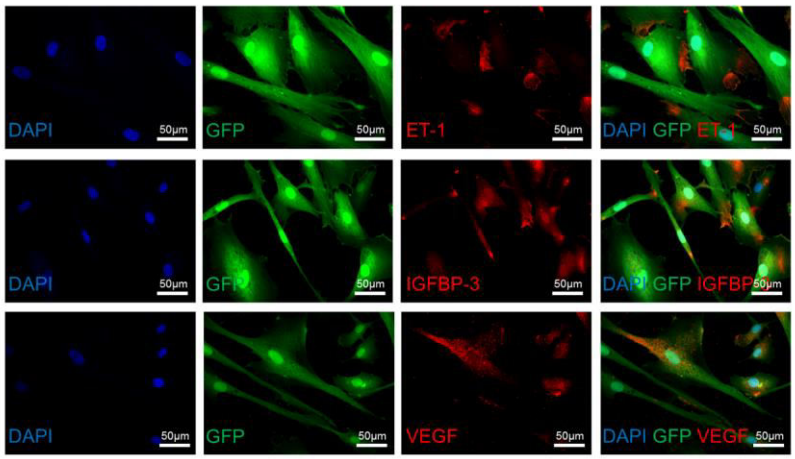
b.

Table 1: Angiogenic growth factor concentrations

	Neg Contr	Pos Contr	CASC CM	CASC lysate
ET-1 (ng/ml)	<<	<<	32.4±9.8	1.2±0.4
IGFBP-3 (ng/ml	<<	<<	885.5±158.2	99.1±43.9
VEGF (ng/ml)	<<	0.04	64.8±18.2	0.8±0.1

<< below detection limit
Data are expressed as mean±SEM

c.



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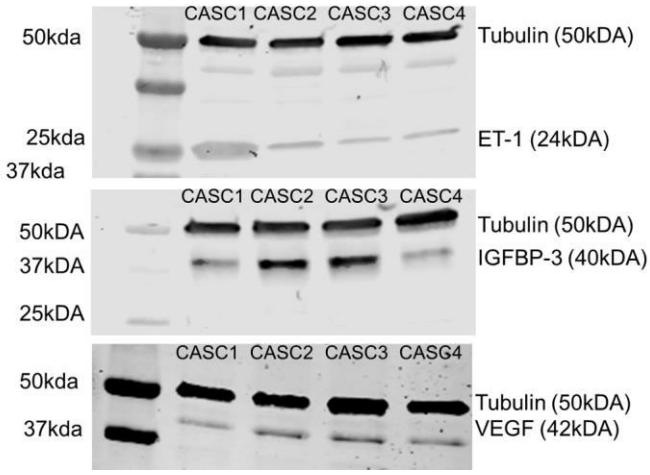

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Figure 1: Angiogenic growth factor expression and secretion by CASCs.

Both anti-angiogenic (red) and pro-angiogenic (green) growth factors were identified in CASC CM in an angiogenesis protein array, while these were absent in the negative control (a; Neg Contr n=1, CASC CM n=3). Relative values were calculated against positive control spots, which were considered as 100% (blue).

Exact concentrations of ET-1, IGFBP-3 and VEGF, measured by ELISA, showed high levels in CASC CM and CASC lysates, but not in the negative control (b; Neg and Pos Contr n=1; CASC CM n=10; CASC lysates n=4). Expression of ET-1, IGFBP-3 and VEGF was confirmed in CASCs by immunofluorescence (c; n=3) and western blot (d; n=4). Data are expressed as mean±SEM.

ANG angiogenin, Ang-1 angiopoietin-1, DPPIV dipeptidyl peptidase 4, GM-CSF granulocyte-monocyte colony stimulating factor, ET-1 endothelin 1, IGFBP-1/2/3, insulin-like growth factor binding protein 1/2/3, IL-8 interleukin 8, MCP-1 monocyte chemotactic protein 1, PTX-3 pentraxin 3, TIMP-1 tissue inhibitor of metalloproteinase 1, uPA urokinase plasminogen activator, VEGF vascular endothelial growth factor.

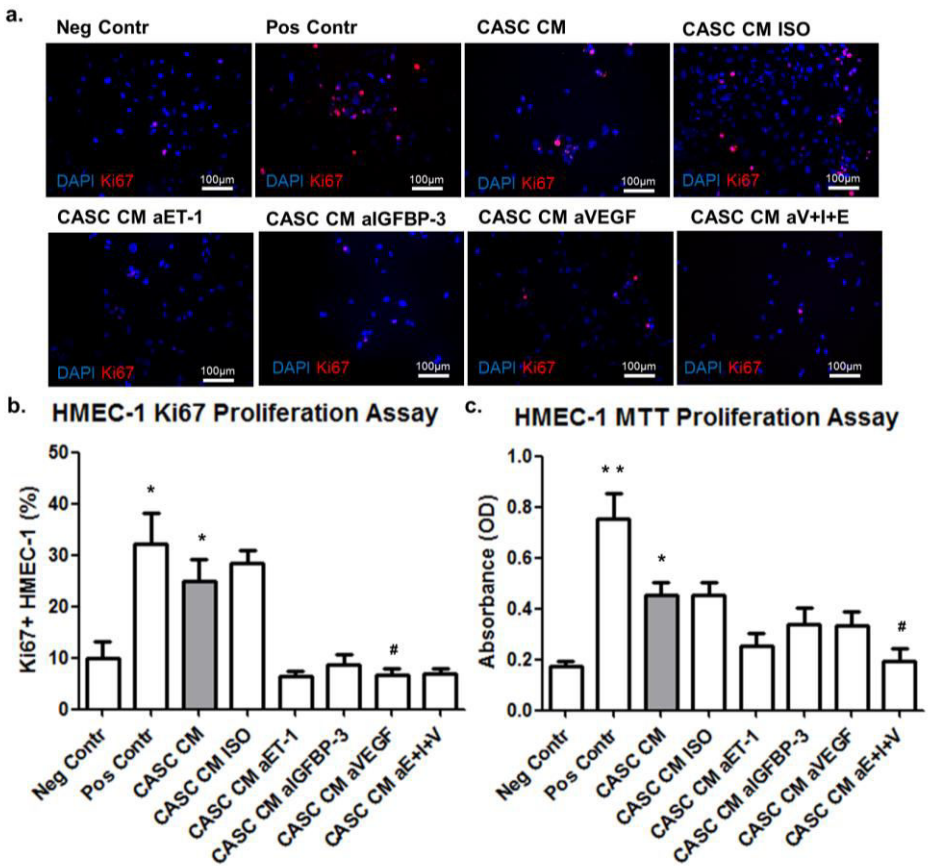

Figure 2: CASCs promote HMEC-1 proliferation in an MTT and a Ki67 assay.

HMEC-1 were cultured in control media and CASC CM for 72h, after which proliferation was examined. Representative pictures of the Ki67 assay are displayed in a with nuclei in blue (DAPI) and Ki67 in red. Both the Ki67 and the MTT assay revealed a significant increase in HMEC-1 proliferation for the positive control and CASC CM compared to the negative control (b,c). This effect was significantly inhibited by an anti-VEGF antibody in the Ki67 assay (b) and by a combination of an antibody against ET-1, IGFBP-3 and VEGF in the MTT test (c). Data are expressed as mean±SEM. The MTT assay was repeated 8 independent times with control media n=11, CASC CM n=18, ISO n=9, aET-1 n=6, aIGFBP-3 n=8, aVEGF n=8, aE+I+V n=6. The Ki67 assay was performed 7 times with control media n=10, CASC CM n=15, ISO n=8, aET-1 n=6, aIGFBP-3 n=6, aVEGF n=6, aE+I+V n=6. * significance compared to Neg Contr; # compared to CASC CM. * or # p-value <0.004; ** p-value < 0.0008. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr medium with 10% FBS, VEGF vascular endothelial growth factor.

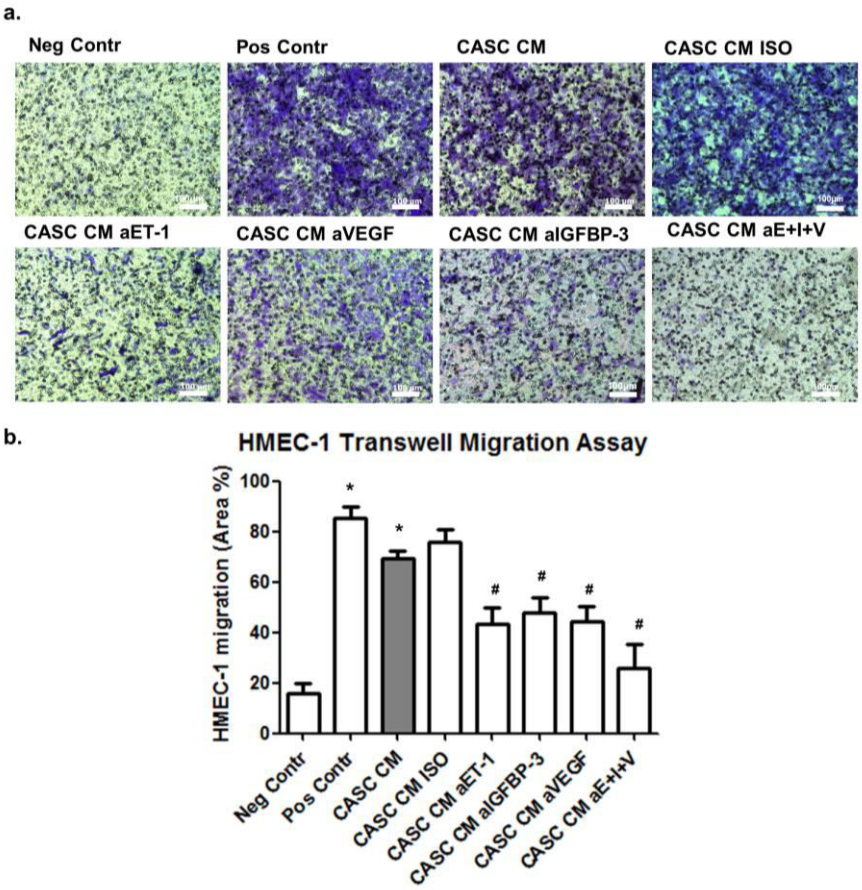

Figure 3: CASCs promote HMEC-1 migration in a transwell assay.

Representative pictures show HMEC-1 migrating through the transwell membrane (A; purple). A significant increase in HMEC-1 migration was observed for the positive control and CASC CM compared to the negative control (B). CM-induced migration could be reduced by addition of antibodies against ET-1, IGFBP-3 and VEGF. Data are expressed as mean±SEM. The transwell assay was repeated 16 independent times with Neg Contr n=17, Pos Contr n=20, CASC CM n=28, ISO n=3, aET-1 n=20, aIGFBP-3 n=18, aVEGF n=15, aE+I+V n=9. * significance compared to Neg Contr; # compared to CASC CM .* or # p-value <0.004. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr medium with 10% FBS, VEGF vascular endothelial growth factor.

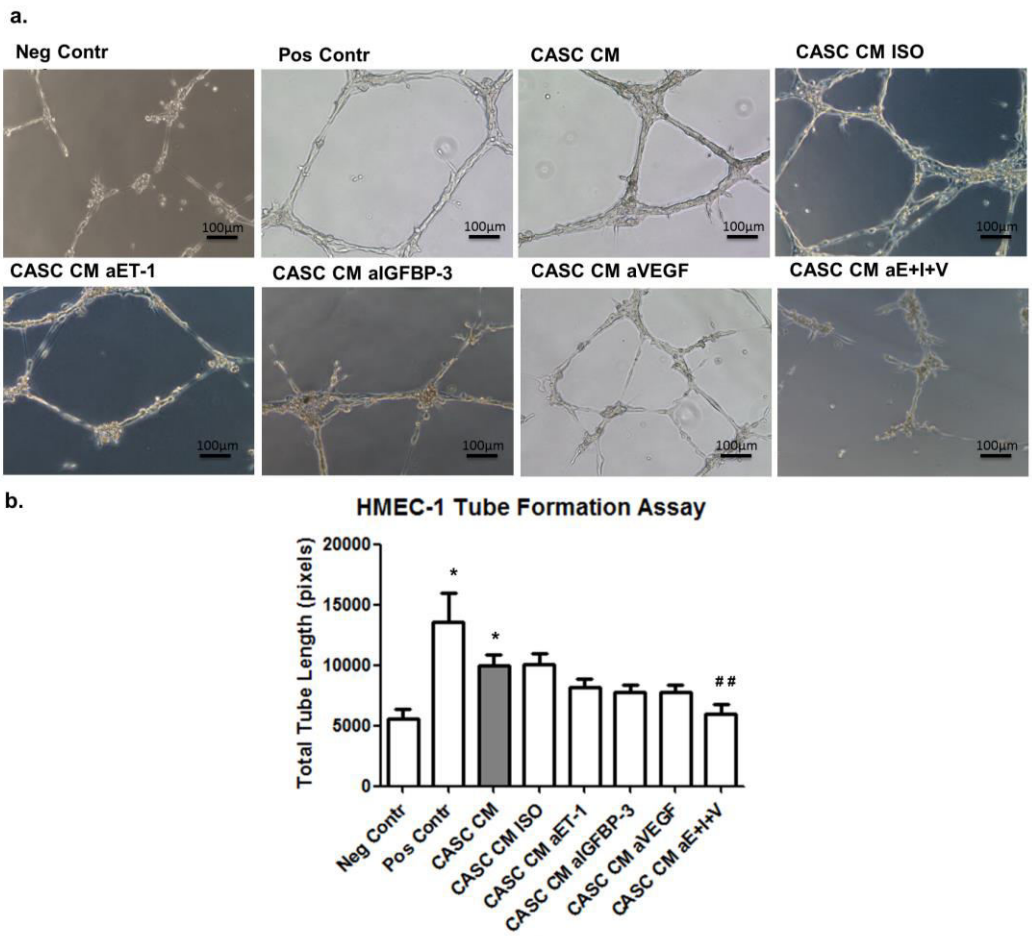

Figure 4: CASCs promote HMEC-1 tube formation.

Representative pictures of HMEC-1 tubular networks after 24h (A). A significant increase in total tube length was observed for the positive control and CASC CM compared to the negative control (B). Combined inhibition of ET-1, IGFBP-3 and VEGF significantly reduced these effects. Data are expressed as mean±SEM. For the tube formation assay, 14 experiments were performed with Neg Contr n=18, Pos Contr n=14, CASC CM n=25, ISO n=6, aET-1 n=17, aIGFBP-3 n=20, aVEGF n=20, aE+I+V n=13. * significance compared to Neg Contr; # compared to CASC CM.* or # p-value <0.004; ## p-value < 0.0008. ET-1 endothelin 1, IGFBP-3, insulin-like growth factor binding protein 3, ISO isotype control antibodies, Neg Contr unconditioned serum-free medium, Pos Contr HMEC-1 culture medium with 10% FBS L-Glut hEGF HC, VEGF vascular endothelial growth factor.

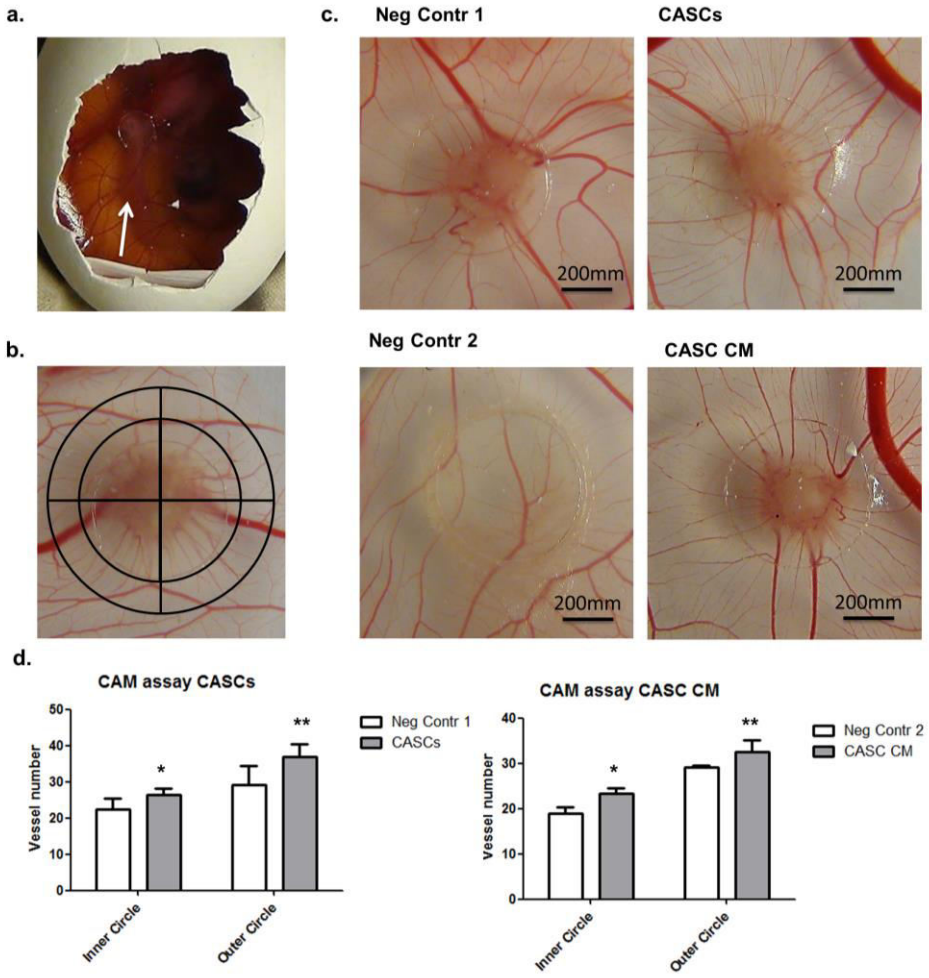

Figure 5: CASCs promote angiogenesis *in vivo* in the CAM assay.

The CAM membrane of fertilized chicken eggs was incubated at E9 with Matrigel droplets containing different test conditions (A). Representative pictures of each condition show the radial ingrowth of blood vessels with a typical spoke wheel pattern (B,C). A significant increase in blood vessel number was observed for CASCs (D, left panel) and CASC CM (D, right panel) compared to their negative control for the inner and outer circle. Data are expressed as mean±SEM. The CAM assay was repeated 3 independent times with 6 CASC and CASC CM donor samples. * p-value <0.05; ** p-value < 0.01. Neg Contr 1 = Matrigel; Neg Contr 2 = Matrigel mixed 1:1 with serum-free medium.